



(11) EP 1 029 920 A1

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 158(3) EPC

(43) Date of publication:
23.08.2000 Bulletin 2000/34

(51) Int. Cl.⁷: C12N 15/57, C12N 9/54,
C12N 1/21, C11D 3/386

(21) Application number: 98947770.8

(86) International application number:
PCT/JP98/04528

(22) Date of filing: 07.10.1998

(87) International publication number:
WO 99/18218 (15.04.1999 Gazette 1999/15)

(84) Designated Contracting States:
DE DK FR GB NL

(30) Priority: 07.10.1997 JP 27457097

(71) Applicant: Kao Corporation
Tokyo 103-8210 (JP)

(72) Inventors:

- TAKAIWA, Mikio,
Kao Corporation Research Lab.
Haga-gun, Tochigi 321-3497 (JP)
- OKUDA, Mitsuyoshi,
Kao Corporation Research Lab.
Haga-gun, Tochigi 321-3497 (JP)
- SAEKI, Katsuhisa,
Kao Corporation Research Lab.
Haga-gun, Tochigi 321-3497 (JP)
- KUBOTA, Hiromi,
Kao Corporation Research Lab.
Haga-gun, Tochigi 321-3497 (JP)

- HITOMI, Jun,
Kao Corporation Research Lab.
Kashima-gun, Ibaraki 314-1103 (JP)
- KAGEYAMA, Yasushi,
Kao Corporation Research Lab.
Haga-gun, Tochigi 321-3497 (JP)
- SHIKATA, Shitsuw,
Kao Corporation Research Lab.
Wakayama-shi, Wakayama 640-8580 (JP)
- NOMURA, Masafumi,
Kao Corporation Research Lab.
Wakayama-shi, Wakayama 640-8580 8580 (JP)
- HITOMI, Jun
2606 Akabane Haga-gun Tochigi 321-3497 (JP)

(74) Representative:
VOSSIUS & PARTNER
Siebertstrasse 4
81675 München (DE)

(54) **ALKALINE PROTEASE**

(57) An alkaline protease having the following properties; a gene encoding the same; a microorganism producing the same; and washing compositions containing the same: (i) acting over a broad pH value range of 4 to 13 and achieving, at pH 6 to 12, an activity 80% or more as high as the one at the optimum pH value; (ii) when treated at 40°C for 30 minutes, being stable over a pH value range of 6 to 11; (iii) having an isoelectric point of about 8.9 to 9.1; and (iv) being free from inhibition by oleic acid on the casein digesting activity. Because of being highly stable to various surfactants, being tolerant to fatty acids and showing a high stability to oxidizing agents, this alkaline protease is useful as an enzyme to be used in cleansers for automatic dish washers and a detergent for clothes, both containing bleaching components.

EP 1 029 920 A1

Description

Technical Field

5 [0001] The present invention relates to an alkaline protease useful as an enzyme incorporated in a detergent; a gene encoding the same; a microorganism producing the same; and a detergent composition containing the same.

Background Art

10 [0002] Protease has been widely used in a variety of detergents, such as laundry detergents; cosmetic compositions; bath additives; food-modifying agents; and pharmaceuticals such as digestive aids and antipthologistics.

[0003] Of these, proteases used in detergents are produced in largest amounts on an industrial scale and thus account for a significant part of commercial supply. Examples of such proteases include Alcalase, Savinase (product of Novo Nordisk), Maxacal (product of Genencor), Blap (Product of Henkel), and Protease K (KAP, product of Kao Corporation).

15 [0004] Meanwhile, attempts have been made to improve the performance of enzymes used in detergents. For example, Japanese Patent Application Laid-Open (*kokai*) No. 6-70765 discloses an enzyme having high stability to heat and a surfactant. Japanese Patent Application Laid-Open (*kokai*) No. 9-121855 discloses an enzyme which acts on insoluble proteins such as keratin and has a high specific activity. Japanese Patent Application Laid-Open (*kokai*) Nos. 5-211868 and 9-121856 disclose an enzyme having excellent activity in a low temperature range. European Patent No. 0130756 discloses a method for enhancing stability of an enzyme to an oxidizing agent.

20 [0005] In many cases, soils on laundry comprise a plurality of components such as lipids and solid particles other than protein. Therefore, there is demand for a detergent having excellent detergency to such complex soils. In order to meet the demand, generally a plurality of enzymes and surfactants have been incorporated into a detergent.

25 [0006] However, even though a plurality of enzymes are incorporated, their effects cannot be fully exerted if, in the presence of complex soils, the enzymes are unstable and do not exhibit constant and sufficient activity. Conventional enzymes are unsatisfactory in this point.

Disclosure of the Invention

30 [0007] In view of the foregoing, the present inventors have discovered an alkaline protease which has a constant casein-degrading activity even in the presence of a fatty acid at a high concentration and exhibits excellent detergency even under complex soil conditions; e.g., soils containing protein and sebum.

[0008] Accordingly, in one aspect of the present invention, there is provided an alkaline protease which has the following physicochemical properties:

(i) Acting pH range

40 acting over a wide pH range of 4-13 and exhibiting, at a pH of 6-12, 80% or more the activity at the optimum pH;

(ii) Stable pH range

being stable over a pH range of 6-11 when treated at 40°C for 30 minutes;

(iii) Isoelectric point

45 having an isoelectric point of approximately 8.9-9.1; and

(iv) Effect of a fatty acid

50 casein-degrading activity not being inhibited by oleic acid.

[0009] In another aspect of the present invention, there is provided a gene encoding the above-described alkaline protease.

55 [0010] In still another aspect of the present invention, there is provided a microorganism producing the above-described alkaline protease.

[0011] In yet another aspect of the present invention, there is provided a detergent composition containing the above-described alkaline protease.

Brief Description of the Drawings

[0012]

Fig. 1 shows the effects of pH on the activity of alkaline protease KP43. Fig. 2 shows the effects of pH on the stability of alkaline protease KP43 (40°C, 30 minutes). Fig. 3 shows the effects of pH on the stability of alkaline protease KP43 (10°C, 24 hours). Fig. 4 shows the effects of temperature on the activity of alkaline protease KP43. Fig. 5 shows the effects of temperature on the stability of alkaline protease KP43. Fig. 6 shows the effect of an oxidizing agent (50 mM hydrogen peroxide) on the activity of alkaline protease KP 43. Fig. 7 shows N-terminal sequences of KP9860 protease and partially degraded products thereof. Fig. 8 shows primer sequences designed from an N-terminal sequence of KP9860 protease. Fig. 9 shows 57 bp PCR-amplified fragments and primer designs.

Best Mode for Carrying Out the Invention

[0013] The alkaline protease of the present invention has the above-described physicochemical properties (i) through (iv). Of these, property (iv) is particularly important. The alkaline protease has a casein-degrading activity in the presence of 10 mM of oleic acid, a component of sebum, as high as that in the absence of oleic acid.

[0014] The alkaline protease of the present invention preferably has (v) an estimated molecular weight of approximately 43,000 as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

[0015] Particularly preferred is an alkaline protease having, in addition to properties (i) through (v), properties (vi) through (ix) as described below.

(vi) Acting temperature and optimum temperature

acting at an optimum temperature of 60°C-70°C, and also acting at a temperature as low as 20°C or lower;

(vii) Effects of metal ions

activity being inhibited by Hg^{2+} and Cu^{2+} and thermal stability being enhanced by Ca^{2+} ;

(viii) Effects of inhibitors

activity not being inhibited by ethylenediaminetetraacetic acid (EDTA) and p-chloromercuribenzoic acid (PCMB) and activity being inhibited by diisopropyl fluorophosphate (DFP) and phenylmethanesulfonyl fluoride (PMSF); and

(ix) Effects of surface active agents

activity not being inhibited by linear sodium alkylbenzenesulfonate, sodium polyoxyethylene alkyl sulfate, sodium dodecyl sulfate, sodium α -olefinsulfonate, or α -sulfotatty acid ester.

[0016] The alkaline protease of the present invention preferably has an amino acid sequence shown by Sequence No. 1 or 2, or such a sequence in which one or more amino acids are deleted, substituted, or added. Sequence No. 1 differs from Sequence No. 2 in that lysine at the 3rd position in Sequence No. 2 is deleted. Xaa in Sequence Nos. 1 and 2 refers to an arbitrary amino acid. Preferable amino acids for Xaa at each position in Sequence No. 2 are shown in the following Table.

Table

position		Position	
24	Ser or Asn	30	Gly or Asp
33	Asn or Thr	47	Ala or Val
48	Lys or Ser	54	Gly or Arg
71	Pro or Leu	75	Gln or Leu
90	Ile or Val	103	Gln or Lys

Table (continued)

position		Position	
106	Lys or Thr	129	Lys or Gln
131	Ala or Lys	132	Thr or Val
133	Ser or Arg	134	Thr or Ser
147	Ile or Lys	149	Arg or Lys
161	Glu or Thr	166	Val or Leu
173	Lys or Asn	184	Gln or Glu
188	Phe or Tyr	189	Ala or Val
190	Ile or Ala	195	Leu or His
287	Ser or Ala	307	Gly or Ser
325	Tyr or Phe	370	Gly or Arg
432	Phe or Tyr	502	Ile or Val
532	Ser or Ala	542	Ser or Thr
585	Gln or Arg	592	Thr or Ser
593	Ser or Ala	595	Tyr or Phe
596	Asn or Asp	597	Asp or Asn
612	Ala or Ser	633	Thr or Asn

[0017] Deletions, substitutions, and additions in the alkaline protease of the present invention are not particularly limited. However, the amino acid sequence shown in Sequence No. 1 or 2 is preferably conserved in the amount of 70% or more, more preferably 80% or more, particularly preferably 90% or more.

[0018] Examples of the alkaline proteases include alkaline proteases having an amino acid sequence shown by Sequence No. 3, 4, or 5, or such a sequence in which one or more amino acids are deleted, substituted, or added.

[0019] The alkaline protease of the present invention may be produced by cultivating alkaline protease-producing microorganisms belonging to the genus *Bacillus* and collecting the enzyme from the culture broth. Examples of alkaline protease-producing microorganisms according to the present invention include wild strains belonging to the genus *Bacillus* and a transformant containing a gene encoding a peptide having the above-described amino acid sequence. Examples of the wild strains include KP-43, KP-1790, and KP-9860. Mycological characteristics of these strains are shown below.

Table 1-a

	KP43	KP1790	KP9860
5	A. Morphological characteristics		
	positive	positive	positive
	undefined	undefined	undefined
10	yes	yes	yes
	peritrichous	peritrichous	peritrichous
	flagella	flagella	flagella
	sporogenous,	sporogenous,	sporogenous,
	elliptical,	elliptical,	elliptical,
15	central,	central,	central to
	none	none	terminal,
			swollen
	B. Physiological characteristics		
20	negative	negative	negative
	negative	negative	negative
	can grow at	can grow at	can grow at
	pH 6.2-11.7,	pH 6.2-11.7,	pH 6.2-10.0,
	well grow at	well grow at	well grow at
25	pH 8-10	pH 8.5-10	pH about 9
	cannot grow	cannot grow	cannot grow
	under $\geq 7\%$	under $\geq 7\%$	under $\geq 7\%$
	NaCl	NaCl	NaCl
30	10-40° C	10-40° C	20-40° C
	positive	positive	positive
	negative	negative	negative
35	negative	negative	negative
	positive	positive	positive
	negative	negative	negative
	negative	negative	negative
	positive	positive	positive
40	negative	negative	negative
	positive	positive	positive
	negative	negative	negative
	negative	negative	negative
45	negative	negative	negative
	negative	negative	negative

(continued to Table 1-b)

Table 1-b

		KP43	KP1790	KP9860
5	(p)Acid production from sugar			
	D-Glucose	+	±	+
	L-Arabinose	-	-	-
10	D-Xylose	-	-	-
	D-Mannitol	+	+	+
	D-Galactose	±	-	-
	Sucrose	+	+	+
15	D-Mannose	+	±	+
	Inositol	-	-	-
	D-Sorbitol	+	-	-
	Trehalose	±	+	+
	Lactose	-	-	-
20	Glycerol	-	-	-
	Maltose	+	±	+
	D-Fructose	+	+	+
	Raffinose	-	-	-
25	Melibiose	+	-	-
	Starch	+	+	+

[0020] Based on the above-described mycological characteristics, the three strains were examined by reference to the pertinent descriptions in "Bergey's Manual of Systematic Bacteriology" (Williams & Wilkins Co., 1984), and were considered to belong to the genus *Bacillus*. However, these strains are novel microorganisms in that characteristics of these species do not completely match those of known species belonging to the genus *Bacillus*. Thus, the three strains were deposited with National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, 305-0046, JAPAN) as *Bacillus* sp. KSM-KP43 (FERM BP-6532), *Bacillus* sp. KSM-KP1790 (FERM BP-6533), and *Bacillus* sp. KSM-KP9860 (FERM BP-6534) (Date of original deposit: September 18, 1996).

[0021] In order to produce the alkaline protease of the present invention by use of the above-described strains, the strains are inoculated in a medium containing an assimilable carbon source, a nitrogen source, and essential nutrients and are cultured through a customary method.

[0022] Collection and purification of a target alkaline protease from the thus-obtained culture broth can be performed according to conventional methods applicable to the collection and purification of common enzymes. For example, cells are separated from the culture broth by centrifugation or filtration, and the target alkaline protease can be obtained from the supernatant through a customary purification method. The thus-obtained enzyme liquid may be used as such or may be further purified and crystallized through a known method.

[0023] Alternatively, the alkaline protease of the present invention may be produced through the following steps: obtaining a gene encoding the alkaline protease; preparing a recombinant vector by use of the gene; transforming a host cell by use of the recombinant vector; cultivating the obtained transformant; and collecting the target alkaline protease from the cultured product.

[0024] The gene encoding the alkaline protease of the present invention may be cloned from any of the three above-described strains. Cloning may be performed through known methods. Examples of the methods include (1) the shot gun method comprising preparation of a DNA fragment through complete or partial digestion of chromosomal DNA by use of an appropriate restriction endonuclease; combination of the fragment into a suitable vector; and expression through introduction to *Escherichia coli* or *Bacillus subtilis*, and (2) a method comprising synthesis of an appropriate primer and cloning a target gene through PCR.

[0025] Examples of the nucleotide sequence of the alkaline protease of the present invention are shown in Sequence Nos. 3 to 5. The nucleotide sequence is not limited to Sequence Nos. 3 to 5, and acceptable sequences may

include a nucleotide sequence encoding the amino acid sequence shown in Sequence No. 1 or 2, and a nucleotide sequence encoding such an amino acid sequence in which one or more amino acids are deleted, substituted, or added. Of these, nucleotide sequences represented by Sequence Nos. 3 to 5, or such sequences in which one or more amino acids are deleted, substituted, or added are preferred. In these cases, deletion, substitution, or addition preferably occurs within the above-described variation of amino acid sequence.

[0026] In order to prepare a recombinant vector including the above-described gene encoding an alkaline protease, the gene may be incorporated into an arbitrary vector suitable for expression of the gene in a host of interest. Examples of the vectors include pUC18, pBR322, and pUC19 in the case in which *Escherichia coli* serves as a host and pUB110 in the case in which *Bacillus subtilis* serves as a host.

[0027] A host is transformed by use of the thus-obtained recombinant vector through a customary method such as the protoplast method or the competent cell method. Although no particular limitation is imposed on the host, microorganisms are preferred. Examples include Gram-positive bacteria such as microorganisms belonging to the genus *Bacillus*, Gram-negative bacteria such as *Escherichia coli*, yeast belonging to *Saccharomyces*, and fungus belonging to *Aspergillus*.

[0028] In order to produce the alkaline protease of the present invention through culturing of the obtained transformant, cultivation, collection, and purification may be performed in accordance with a procedure employed in the case in which the above-described wild strain is used.

[0029] As described above, the alkaline protease of the present invention has excellent resistance to alkaline conditions and excellent protease activity even in the presence of lipids. Thus, the alkaline protease is useful for an enzyme incorporated in a variety of detergent compositions.

[0030] No particular limitation is imposed on the amount of the above-described alkaline protease incorporated into a detergent composition, and the amount is preferably 0.1-5000 U based on 1 kg, particularly preferably 1-500 U, of the detergent composition.

[0031] Known detergent components may be incorporated into the detergent composition of the present invention containing the alkaline protease. For example, components described in WO94/26881 (p. 5, upper-right column, line 14 - lower-right column, line 29) may be employed.

[0032] A surfactant is incorporated into the detergent composition in an amount of 0.5-60 wt.% (hereinafter simply referred to as "%"), particularly preferably 10-45%, into a powdery detergent composition and in an amount of 20-50% into a liquid detergent composition. When the detergent composition of the present invention serves as a bleaching detergent composition or a detergent composition for an automated dishwasher, a surfactant is typically incorporated in an amount of 1-10%, preferably 1-5%.

[0033] A divalent metal ion scavenger is incorporated in an amount of 0.01-50%, preferably 5-40%.

[0034] An alkali agent and an inorganic salt are incorporated in an amount of 0.01-80%, preferably 1-40%.

[0035] An anti-redeposition agent is incorporated in an amount of 0.001-10%, preferably 1-5%.

[0036] The detergent composition may contain an enzyme other than the alkaline protease of the present invention. Examples include cellulase, amylase, protopectinase, pectinase, lipase, hemicellulase, β -glucosidase, glucose-oxidase, and cholesterol-oxidase. These enzymes are incorporated in an amount of 0.001-5%, preferably 0.1-3%.

[0037] A bleaching agent such as hydrogen peroxide or percarbonate is preferably incorporated in an amount of 1-10%. When a bleaching agent is incorporated, a bleach-activator may be incorporated in an amount of 0.01-10%.

[0038] Examples of fluorescent agents incorporated into the composition include a biphenyl compound, such as Cinopearl CBS-X, and a stilbene compound such as DM-type fluorescent agent. The fluorescent agent is preferably incorporated in an amount of 0.001-2%.

[0039] The above-described detergent composition may be processed into a variety of forms such as liquid, powder, and granules. The detergent composition may be used for laundry, an automated dishwasher, drain pipes, and dentures, and may be used as a bleaching agent.

Examples

Example 1 (screening for alkaline protease-producing microorganisms)

[0040] A soil sample (1 g) was suspended in physiological saline (10 ml) and thermally treated at 80°C for 10 minutes, followed by inoculation in liquid enrichment medium for protease-producing microorganisms, the medium having the following composition, to thereby culture at 20°C. After subculture enrichment was repeated about three times in the same medium, the cultivated product was smeared onto a plate for judging protease-production and cultivated at 20°C for 5-7 days. Colonies around which a transparent zone was formed by dissociation of skim milk were selected for collection of protease-producing microorganisms. By means of the above procedure, the *Bacillus* sp KSM-KP43 strain, the KSM-KP1790 strain, and the KSM-KP9860 strain were obtained as alkaline protease-producing microorganisms.

Table 2

Composition of liquid enrichment medium for screening (pH 11)	
Monopotassium phosphate	0.1%
Magnesium sulfate	0.02%
Yeast extract (Difco)	0.05%
Keratin (Tokyo Kasei)	1.0%
Glucose	0.5%
Sodium carbonate	0.3%
Agar plate medium for screening	
Nutrient agar (Difco)	2.3%
Skim milk (Difco)	0.3%
Sodium carbonate	1.0%

Example 2

[0041] The *Bacillus* sp KSM-KP43 strain obtained in Example 1 was inoculated in a liquid medium comprising polypeptone S (1%), yeast extract (0.05%), potassium phosphate (0.1%), magnesium sulfate (0.02%), glucose (separately sterilized) (1%), and sodium carbonate (separately sterilized) (0.5%) to thereby be cultivated at 30°C for 24 hours. The concentration of enzyme in the supernatant liquid was about 1.5 U/L. The supernatant liquid which had been centrifugally separated from cells at 4°C was added with pulverized ammonium sulfate under stirring so as to attain 90% of saturated concentration. The solution was maintained under stirring at 4°C for an entire day and night and the resultant precipitate was centrifugally collected. The obtained precipitate was dissolved in 10 mM of a Tris-hydrochloric acid buffer solution (pH 7.5) containing 5 mM of calcium chloride, followed by dialysis through the buffer solution. Subsequently, the dialyzed liquid was applied to a DEAE-Sepharose FF column (product of Pharmacia) which had been equilibrated with 10 mM of a Tris-hydrochloric acid buffer solution (pH 7.5) containing 5 mM of calcium chloride, to thereby collect the non-absorbed fraction. The fractionated liquid was dialyzed through 50 mM of HEPES buffer solution (pH 7.5) containing 2 mM of calcium chloride and was applied to a SP-Sepharose FF column which had been equilibrated with the same buffer solution, to thereby collect an active fraction which has eluted slightly after the non-absorbed fraction. While the active fraction, which had a recovery ratio of 15%, was used as a sample, SDS-polyacrylamide electrophoresis was carried out, and as a result, a single band was obtained for the respective enzyme.

Example 3

[0042] The obtained *Bacillus* sp KSM-KP1790 strain and KSM-KP9860 strain were cultivated in the same medium as in Example 2 and the alkaline protease was purified in the same manner as in Example 2.

Example 4

[0043] Enzymatic properties of the alkaline proteases obtained in Example 2 and 3 were examined. The methods and results of the experiments are described below.

I. Materials and methods for experiments

(1) Methods for activity measurement

(a) Method in which casein is used as a substrate

[0044] After 1 mL of 50 mmol/L of various buffer solutions containing 1% (w/v) Casein (Hammerstein: product of Merck Inc.) was maintained at 40°C for 5 minutes, 0.1 mL of an enzyme solution was added to the solution, followed by incubation at 40°C for 10 minutes. 2 mL of a TCA solution (0.11 mol/L trichloroacetic acid : 0.22 mol/L sodium acetate

: 0.33 mol/L acetic acid) was added to stop the reaction and the mixture was left to stand at room temperature for 10 minutes. Subsequently, acid-denatured protein was filtered (No. 2 filter paper: product of Whatmann). To 0.5 mL of the filtrate, 2.5 mL of alkaline copper reagent (1% (w/v) sodium potassium tartrate : 1% (w/v) copper sulfate : 2% (w/v) sodium carbonate, 0.1 mol/L sodium hydroxide = 1:1:100 (v/v)) was added, and after the solution was maintained at 30°C for 10 minutes, 0.25 mL of diluted phenol reagent (phenol reagent (product of Kanto Chemical) diluted two-fold with deionized water) was added, and after being maintained at 30°C for 30 minutes, the solution was subjected to an absorbance measurement at 660 nm. The following solution was used as a blank: to the above-described system of enzyme reaction, a reaction termination solution was mixed and then the enzyme solution was added.

[0045] One unit (P.U) of enzymatic activity was defined as the amount of enzyme that released acid-soluble protein degradation products equivalent to 1 mmol of tyrosine per minute under the above reaction conditions.

(b) Method in which Synthetic oligo-peptide is used as a substrate

[0046] 0.05 mL of 50 mmol/L synthetic oligo-peptide solution (succinyl-alanyl-alanyl-prolyl-leucine para-nitroanilide dissolved in dimethyl sulfoxide) was mixed into 0.9 mL of 100 mmol/L boric acid buffer solution (pH 10.0, containing 2 mmol/L of calcium chloride), and after the solution was maintained at 30°C for 5 minutes, 0.05 mL of an enzyme solution was added, followed by incubation at 30°C for 10 minutes. 2 mL of 5% (w/v) citric acid was added to stop the reaction and absorbance at 420 nm was measured.

[0047] One unit (U) of enzymatic activity was defined as the amount of enzyme that released acid-soluble protein degradation products equivalent to 1 mmol of tyrosine per minute under the above reaction conditions.

(c) Method in which Hemoglobin is used as a substrate

[0048] According to the method by Anson (M. L. Anson, J. Gen. Physiol. 22, 79(1983)), hemoglobin of bovine blood serum was denatured by use of urea and adjusted to pH 10.5 with sodium hydroxide. 0.1 mL of an enzyme solution (1.0×10^{-5} - 1.0×10^{-3} A.U) was added to 0.5 mL of the substrate solution (2.2% in terms of hemoglobin), and the resultant solution was incubated at 25°C for 10 minutes. To the resultant solution, 1.0 mL of 4.9% trichloroacetic acid was added to stop the reaction. After completion of the reaction, centrifugation (3,000 rpm, 10 minutes) was carried out and protein degradation products in the supernatant liquid were quantitatively determined according to the Folin-Lowry method (O. H. Lowry *et al.*, J. Biol. Chem., 193, 265(1951)).

[0049] One unit (A. U) of enzymatic activity was defined as the amount of enzyme that released acid-soluble protein degradation products equivalent to 1 mmol of tyrosine per minute under the above reaction conditions.

(2) Optimum pH

[0050] 0.1 mL of an enzyme solution (3.0×10^{-5} mP. U) was added to 1 mL of 50 mmol/L Britton-Robinson buffer solution containing 1% (w/v) casein, and activity was measured according to the casein method.

(3) pH stability

[0051] An enzyme solution (8.0×10^{-4} mP. U.) was mixed into Britton-Robinson buffer solution (20 mmol/L, containing 2 mmol/L calcium chloride), followed by treatment at 40°C for 30 minutes or at 10°C for 24 hours. After ice-cooling, the treated solution was diluted 40-fold with 50 mmol/L boric acid buffer solution, followed by measurement of residual activity according to the method in which casein is used as a substrate.

(4) Optimum temperature

[0052] 0.1 mL of the enzyme solution (2.0×10^{-5} mP. U.) was added to 1 mL of 50 mmol/L boric acid buffer solution (pH 10.0) containing 1% (w/v) casein, and activity of the enzyme was measured at temperatures between 10-80°C according to the casein method.

[0053] The activity measurements were conducted in both systems; i.e., in the presence of and in the absence of 5 mmol/L calcium chloride.

(5) Heat stability

[0054] An enzyme solution (2.5×10^{-4} mP. U.) was added to 20 mmol/L boric acid buffer solution (pH 10.0) in both systems; i.e., in the presence of and in the absence of 5 mmol/L calcium chloride, and thermally treated at the appropriate temperature for 10 minutes. After being cooled with ice, the treated solution was diluted 5-fold with 50 mmol/L

boric acid buffer solution (pH 10.0), and residual activity was measured using casein as a substrate.

(6) Effects of metal ions

5 [0055] An enzyme solution (4.0×10^{-4} mP. U.) was added to 20 mmol/L boric acid buffer solution (pH 10.0) containing 1 mmol/L various metal salts, and the resultant solution was incubated at 30°C for 20 minutes. The solution was diluted 5-fold with 50 mmol/L boric acid buffer solution (pH 10.0), followed by measurement of activity using casein as a substrate.

10 (7) Effects of inhibitors

[0056] The enzyme solution (1.0×10^{-3} mP. U.) was added to 10 mmol/L phosphoric acid buffer solution (pH 7.0) containing various inhibitors so as to attain a predetermined concentration, and the solution was incubated at 30°C for 20 minutes. Subsequently, the solution was diluted 20-fold with deionized water, and residual activity was measured using casein as a substrate.

(8) Effects of surfactants

20 [0057] An enzyme solution (7.0×10^{-4} mP. U.) was added to 100 mmol/L boric acid buffer solution containing dissolved surfactants in an amount of 1%, and the resultant solution was incubated at 40°C for 4 hours. The solution was diluted 20-fold with 50 mmol/L boric acid buffer solution (pH 10.0), and residual activity was measured using casein as a substrate.

(9) Effects of oxidizing agent (hydrogen peroxide)

25 [0058] 2.7 mL of Britton-Robinson buffer solution containing hydrogen peroxide and calcium chloride (final concentration: 50 mmol/L hydrogen peroxide, 2 mmol/L calcium chloride, 20 mmol/L Britton-Robinson) (pH 8.0) was maintained at 30°C for 15 minutes, and then 0.3 mL of an enzyme solution was added. With the passage of time, 0.8 mL of the resultant solution was sampled in a previously prepared test tube containing 5 μ L of catalase (Boehringer Mannheim Co.: 20 mg/L), to thereby stop the oxidation reaction. Each sample was suitably diluted with 2 mmol/L calcium chloride, and residual activity was measured according to the method in which synthetic oligo-peptide is used as a substrate.

(10) Effects of fatty acids

35 [0059] By use of 50 mM phosphoric acid buffer solution (pH 7) containing 1% (w/v) casein as a substrate solution, a reaction was carried out in the presence of 0-10 mM sodium oleate at 20°C for 15 minutes, and activity was measured using casein as a substrate.

40 II. Results

(1) Optimum pH

45 [0060] Effects of pH on three kinds of protease (KP43, KP1790, and KP9860) were examined. Fig. 1 shows the activities of KP43 at each pH value normalized with respect to activity at optimum pH (100%), indicating that the optimum working pH range of the proteases of the present invention is 6-12. Thus, these enzymes exhibit a high protein-degradation activity in the extensively broad working pH range.

(2) pH stability

50 [0061] After being allowed to stand at 40°C for 30 minutes or at 10°C for 24 hours, the residual activity of KP43 was measured over a range of pH values. Figs. 2 and 3 show the residual activities normalized with respect to the enzyme activity before treatment (100%). The results show that the enzymes of the present invention are stable over the pH range of 6-12 after treatment at 40°C for 30 minutes, and that addition of calcium ions improves enzyme stability at pH 5. Similarly, the results show the enzymes of the present invention are stable over the broad pH range of 5-12 after treatment at 10°C for 24 hours.

(3) Optimum temperature

[0062] By use of casein as a substrate, the effects of temperature on the proteases were examined. Fig. 4 shows the activities of KP43 over a range of temperatures, normalized with respect to the highest activity in the absence of calcium ions (100%). The results indicate that in the absence of calcium ions the optimum temperature is 60°C, and in the presence of calcium ions the optimum temperature is 70°C for all three kinds of proteases. Therefore, the results show that the optimum temperature is shifted upward by addition of calcium ions, as is the case with conventional proteases for a detergent.

(4) Heat stability

[0063] Heat treatment was carried out for 10 minutes at temperatures in the range of 30-80°C (pH 10.0, in the presence of and in the absence of 5 mmol/L calcium chloride), and residual activity was measured. Fig. 5 shows residual activity of KP43 at each treatment temperature, normalized with respect to the activity before treatment (100%). The results indicate that the proteases are stable at the temperature up to 60°C in the absence of calcium chloride, and that addition of calcium chloride (5 mmol/L) has the effect of shifting temperature stability upward about 10°C. In comparison with commercially available detergent enzymes, these enzymes have high temperature stability; namely, stability comparable to that of Esperase, which exhibits the most excellent temperature stability among commercially available enzymes.

(5) Effects of metal ions

[0064] In 20 mmol/L boric acid buffer solution (pH 10), 3 kinds of proteases were treated with various metal salts (1 mmol/L) at 30°C for 20 minutes and the residual activity was measured. Residual activity is normalized with respect to enzyme activity obtained for protease treated in the same manner except without the addition of metal salts (100%) (see Table 3.) The results show that the activity is inhibited by mercury chloride and silver nitrate but that the activity is extremely stable for other metal salts.

Table 3

Metal salt (1 mM)	Residual activity (%)		
	KP43	KP1790	KP9860
not added	100	100	100
AgNO ₃	66	70	45
NiCl ₂	92	95	96
CaCl ₂	97	95	101
CoCl ₂	91	101	98
FeCl ₃	93	113	96
ZnCl ₂	85	94	91
CuCl ₂	91	96	94
HgCl ₂	38	37	33
MgCl ₂	92	103	100
Treatment conditions: 1 mM metal salt, 20 mM borate buffer (pH 10.0) 30°C, 20 minutes			

(6) Effects of various inhibitors

[0065] Effects of general enzyme inhibitors on the alkaline proteases of the present invention were examined. A variety of inhibitors were added to 10 mmol/L phosphoric acid buffer solution (pH 7.0) so as to attain the predetermined concentration, and the resultant solution was incubated at 30°C for 20 minutes, after which residual activity was measured. The residual activity is normalized with respect to the enzyme activity obtained for protease treated in the same manner as described above in the absence of inhibitors (100%) (refer to Table 4). The results indicate that for all three

kinds of proteases activity was inhibited by diisopropyl fluorophosphoric acid (DFP), phenylmethanesulfonyl fluoride (PMSF), and chymostatin, which are known inhibitors of serine protease. Therefore, the proteases of the present invention are considered to have serine residue in its active center. In contrast, effects of actinomycetes-derived antipine and leupeptin, which has been reported to inhibit serine protease, were not found.

Table 4

Inhibitor	Residual activity (%)			
	Concentration(mM)	KP43	KP1790	KP9860
free	-	100	100	100
EDTA	5	110	97	101
EGTA	5	92	91	90
o-Phenanthroline	5	100	103	100
DTT	5	104	102	105
PCMB	1	125	115	126
NEM	5	97	100	100
DFP	1	14	17	16
PMSF	1	0	0	0
Chymostatin	0.1	87	87	80
Antipine	0.1	103	99	97
Leupeptin	0.1	102	101	93
E-64	0.1	104	99	103
Elastatinal	0.1	99	102	102

EDTA: ethylenediaminetetraacetic acid (Sigma)
 EGTA: ethyleneglycoltetraacetic acid (Sigma)
 DTT: dithiothreitol (Sigma)
 PCMB: p-chloromercury benzoate (Sigma)
 NEM: N-ethylmaleimide (Sigma)
 DFP: diisopropylfluorophosphoric acid (Sigma)
 PMSF: phenylmethanesulfonyl fluoride (Sigma)

(7) Effects of surface active agents

[0066] Each protease was treated with a variety of 1% surface active agent at 40°C for 4 hours in 0.1 mol/L Tris-hydrochloride buffer solution (pH 9.0), and residual activity was measured. Residual activity is normalized with respect to the enzyme activity in the case of no treatment (100%) (refer to Table 5.), indicating that the three kinds of enzymes are extremely stable to surfactants typified by linear alkylbenzenesulfonic acid (LAS). Accordingly, the enzymes are considered to be useful as a detergent component containing surfactants.

Table 5

Surfactant (concentration: 1%)	Residual activity		
	KP43	KP1790	KP9860
free	100	100	100
Na linear alkylbenzenesulfonate (LAS)	100	88	100
Na polyoxyethylene alkylsulfate (ES)	101	102	104
Na dodecyl sulfate (SDS)	104	97	103

Table 5 (continued)

Surfactant (concentration: 1%)	Residual activity		
	KP43	KP1790	KP9860
Na α -olefinsulfonate (AOS)	100	111	100
Na alkyl sulfate (AS)	113	107	107
α -Sulfofatty acid ester (α -SFE)	112	113	105
Softanol 70H	109	109	104
Treatment conditions: 1% surfactant, 100 mM borate buffer (pH 10.0) 40°C, 4 hours			

(8) Effects of oxidizing agents

[0067] Each protease was treated at 30°C in 50 mmol/L Britton-Robinson buffer solution containing hydrogen peroxide (pH 8.0), and the residual activity was measured with passage of time. As shown in Fig. 6, KP43 exhibited much greater stability than that of commercially available Savinase or KAP and showed stability as high as that of Durazyme (Novo Nordisk), which was developed by imparting oxidizing agents-resistance to Savinase by use of protein engineering techniques.

(9) Effects of fatty acids

[0068] As shown in Table 6, the activity of alkaline proteases of the present invention was not inhibited by oleic acid, one of the components of sebum.

Table 6

	Relative activity (%) in the presence of fatty acid				
	oleic acid concentration (mM)				
	0	1	2	5	10
KP43 protease	100	100	100	103	119
KP1790 protease	100	100	100	103	121
KP9860 protease	100	100	100	100	106

Example 5 (Cloning of a gene encoding KP9860 protease)

(1) Preparation of genomic DNA of KSM-KP9860

[0069] The KSM-KP9860 strain was cultivated in a liquid medium (0.5% glucose, 0.2% Polypepton-S, 0.05% yeast extract, 0.1% $\text{KH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$, 0.26% NaCO_3 ; pH 9.0) (500 mL) at 30°C for two days, and the cells were collected by centrifugation. Genomic DNA was prepared from the obtained cells by the method of Saito and Miura (*Biochim. Biophys. Act.*, 72, 619(1963)).

(2) Limited proteolysis of KP9860 protease

1) Denaturation of KP9860 protease

[0070]

KP9860 protease (5 mg/mL)	45 μL
---------------------------	------------------

(continued)

PMSF (100 mM)	20 μ L
EDTA (200 mM)	10 μ L
SDS (0.08 mg/mL)	25 μ L

[0071] A protease solution with the above composition was heated in boiling water for 10 minutes. The protease solution was dialyzed against ammonium acetate (2 mM), to thereby remove SDS, EDTA, and PMSF, and was then lyophilized. Subsequently, the lyophilized protease was dissolved in distilled water (100 μ L), to thereby serve as a sample of denatured protein.

2) Limited proteolysis by trypsin

[0072]

Denatured protein sample	100 μ L
Trypsin (1 μ g/mL, Sigma)	100 μ L
1M Tris-HCl (pH 7.5)	50 μ L
Distilled water	750 μ L

[0073] Trypsin was allowed to react against the denatured protein prepared in 1) in an ice bath for 3 hours in the solution with the above composition. After addition of 300 μ L of SDS (0.08 mg/mL), 100 μ L of EDTA (200 mM) and 200 μ L of PMSF (100 mM), limited proteolysis was terminated by heating in boiling water for 3 minutes.

[0074] SDS, EDTA, and PMSF were removed through dialysis against ammonium acetate (2 mM), and the solution was lyophilized. Subsequently, the lyophilized was dissolved in distilled water (100 μ L), to thereby serve as a sample for SDS-PAGE.

3) Recovering of the partially degraded product

[0075] The sample obtained in 2) was subjected to SDS-PAGE with 12% Ready-gel-J (product of Bio-Rad). Protein bands were detected through staining with quick CBB staining solution (product of Bio-Rad). The gel containing the protein band was cut with a razor, and the gel slice was crushed into pieces in a 1.5-mL tube. The buffer for SDS-PAGE (composition: glycine 14.4% (W/V), Tris 3.03%, SDS (product of Bio-Rad) 10%) was added in 5 volumes of the crushed gel, and the mixture was stirred at room temperature, to thereby elute the protein band. The eluate was dialyzed against ammonium acetate (2 mM) and was then lyophilized. The lyophilized sample was served to determine the N-terminal sequence for Protein Sequence type 476A (product of Applied Biosystem).

[0076] The obtained N-terminal sequences are shown in Fig. 7.

(3) PCR

[0077] 20-30 Nucleotides primers for 5'-terminal of + chain and that of the - chain corresponding to the obtained N-terminal sequences were synthesized. PCR reaction was carried out in a 100- μ L reaction system by use of a template DNA (100 ng), a primer (20 pmol), and PwoDNA polymerase (product of Boehringer Mannheim). When inverse PCR was performed, Expand™ long template PCR system (product of Boehringer Mannheim) was used in a 50- μ L reaction system. PCR carried out by use of these primers, 9860-N2 and 9860-25k-RV, provided a DNA fragment of 527 bp.

(4) Subcloning of the PCR product

[0078] The PCR product was purified with a High Pure PCR Product Purification Kit (product of Boehringer Mannheim) and inserted to the *Sma* I site of pUC18 through overnight reaction at 16°C with Ligation kit ver. 2 (product of Takara). The resultant recombinant plasmid and the competent cell *E. coli* JM109 strain (product of Takara) were mixed, and the mixture was subjected to heat shock (42°C, 45 seconds), to thereby transform the *E. coli* JM109 cells. LB was added to the cells. After being maintained at 37°C for one hour, the mixture was applied to an LB plate containing IPTG

(0.1 mM, Sigma), X-gal [0.004% (w/v), Sigma], and ampicillin (50 µg/mL, Sigma). Cultivation was performed overnight at 37°C, and grown white colonies were selected as transformants having the recombinant plasmid.

(5) Determination of the nucleotide sequence

[0079] The transformant was cultivated overnight at 37°C in LB containing ampicillin (50 µg/mL), and cells were collected through centrifugation. The recombinant plasmid was obtained by use of High Pure Plasmid Isolation Kit (product of Boehringer Mannheim). PCR for sequencing was performed in a 20-µL reaction system by use of a primer and a DNA sequencing kit (product of PERKIN ELMER), the obtained recombinant plasmid (1 µg) was served as a template DNA. The reaction product was purified by use of Quick Spin Column (product of Boehringer mannheim), and dried up by use of a centrifugal evaporator. The thus-treated sample was subjected to analysis by use of DNA Sequencer Type 377 (product of Applied Biosystem).

[0080] The DNA fragment obtained through PCR had the amino acid sequence which matches the N-terminal sequence of the KP-9860 protease, and there were observed sequences, which match common sequences near Asp and His among three amino acids (Asp, His, Ser) forming an active center of alkaline protease such as subtilisin. Thus, the DNA fragment was considered to be a portion of the KP-9860 protease gene.

(6) Southern hybridization

[0081] KP9860 chromosome was treated with *Eco*R I, *Sac* I, *Kpn* I, *Hind* III, *Bam* H I, *Xho* I, *Pst* I, and *Bgl* II. Southern hybridization was performed by use of the obtained 527 bp DNA as a probe, to thereby detect a complementary region.

[0082] As a result, hybridization bands were observed in the lanes other than the lane attributed to *Kpn* I.

(7) Inverse PCR

[0083] Inverse PCR was performed by use of primers (1 ~ 4 (Fig. 9) Synthesized from the obtained 527 bp sequence. The KP-9860 chromosome was completely digested by use of restriction enzymes, i.e., *Eco*RI, *Hind*III, *Pst*I, and *Bgl*II, and each sample was treated by use of Ligation Kit Ver. 2 (product of Takara) for circularization. Each of the resultant reaction mixtures was served as a template DNA for inverse PCR. PCR reaction (conditions; (94°C-10 seconds, 60°C-30 seconds, 68°C-4 minutes) × 10 cycles; (94°C-10 seconds, 60°C-30 seconds, 68°C-4 minutes + 20 × the number of cycles) × 20 cycles; 68°C-7 minutes; and 4°C-1 minute) was performed by use of the template DNA described above (0.1 µg), primers 1 and 4 (10 pmol, respectively), and the Expand Long Plate PCR System. In addition, PCR (conditions; as described above) was performed by use of the template DNA derived from *Eco*RI digested chromosome (0.1 µg), primers 2 and 3 (10 pmol, respectively), and the Expand Long Plate PCR System. The resultant amplified DNA fragments were purified by use of High Pure PCR Product Purification Kit, and terminals were converted to blunt-ended by use of DNA Blunting Kit (product of Takara). Each of the obtained DNA fragments and *Sma*I digested pUC18 were mixed, and the mixture was treated with Ligation Kit Ver. 2. As described above, *E. coli* JM 109 strain was transformed by the recombinant plasmid, and the obtained recombinant plasmid was served as a template DNA for sequencing. Thus, the nucleotide sequence of the amplified DNA fragments was determined.

(8) Analysis of the entire nucleotide sequence of the KP-9860 protease gene

[0084] The sequencing revealed that the KP-9860 protease gene contains an open reading frame (ORF) encoding the 1917 bp, 639 amino acid residues and that the ORF contains a region (NDVARHIVKADVAQSSYGLY) which matches the N-terminal sequence of the purified KP9860 protease. Judging from the N-terminal sequence, the mature region of KP9860 protease gene was deduced to be the 1302 bp, encoding 434 amino acid residues (Sequence No. 3, molecular weight 45310 Da). Upstream of the ORF, there were observed sequences which are deduced to be a promoter region (-35 region: ttgtgt, -10 region: tacgat) and a ribosome-binding site (SD sequence: aggagt). Downstream of the termination codon (taa), there was an inverted repeat having a free energy of -26.2 kcal/mol, which is deduced to be a terminator.

[0085] The procedure of Example 5 was repeated, to thereby analyze the entire nucleotide sequence and amino acid sequence of each of the genes of KP-43 protease and KP-1790 protease. The results are shown in Sequence Nos. 4 and 5.

Example 6

Washing Test:

5 [0086] A washing test was carried out according to JIS K 3371. Detergents whose compositions are shown in Table 7 were dissolved in water containing 71.2 mg of CaCO_3/L (4°DH) so as to adjust the concentration, and each protease was added to detergent solution so as to adjust the concentration of the alkaline protease to 40 mAPU/L according to the Anson-Hemoglobin method (see Table 8).

10 [0087] Collars of shirts (worn for 3 days) were employed as specimens. For comparison, after the cloth of a collar was cut into a size of about 8×8 cm, the cloth was washed at 15°C and 100 rpm, for 10 minutes by use of a Terg-O-Tometer (Ueshima Seisakusho) with addition of the enzyme or without addition of the enzyme. After being rinsed and dried, pairs of collar clothes (15 pairs) were compared and evaluated by visual judgement. When the soil was almost completely cleaned, an evaluation of 5 was assigned, and when the soil was hardly cleaned, an evaluation of 1 was assigned, and the total scores of 15 specimens were calculated. The detergency index was expressed as the scores of 15 each composition, with the detergency of a detergent composition without addition of the enzyme taken as 100. The results are shown in Table 8.

20

25

30

35

40

45

50

55

Table 7

(wt. %)			
Compound (%)	Detergent A	Detergent B	Detergent C
LAS	23.0	4.0	20.0
AS	4.0		
AE	5.0		
AEP		5.0	
AES		20.0	
Fatty acid salt	3.0	2.5	2.0
Zeolite	22.0		20.0
Sodium carbonate	15.0		
Potassium carbonate	3.0		
Amorphous silicate	7.0		7.0
Crystalline silicate	4.0		
Sodium sulfite	2.0	0.5	2.0
Sodium sulfate	2.0		23.0
AA-MA	5.0		
Citrate			10.0
PEG	2.0		2.0
Monoethanolamine		8.0	
Ethanol		5.0	
Water	3.0	balance	7.0
Form	G*	L**	G*
Concentration in use	20g/30L	20g/30L	40g/30L
pH after washing	10.7	9.2	8.0
LAS: sodium linear alkyl(C12-C14)benzene sulfonate (free acid incorporated into a liquid detergent) AS: alkyl sulfate AE: polyoxyethylene lauryl ether (average EO addition of 4 moles) AEP: polyoxyethylene polyoxypropylene lauryl ether (average EO addition of 8 mol, average PO addition of 3 mol) AES: alkyl ether sulfate (average EO addition of 2.5 mol) Fatty acid: palm oil-derived fatty acid sodium salt Zeolite: zeolite 4A, average particle size of 3 μ m Sodium carbonate: dense ash Amorphous silicate: JIS No. 2 sodium silicate Crystalline silicate: pulverized SKS-6 (product of Hoechst Tokuyama), average particle size of 15 μ m AA-MA: Sokalan CP5, acrylic acid-maleic acid copolymer (product of BASF) PEG: polyethylene glycol, average molecular weight of 8,000			

*) G stands for granular.

**) L stands for liquid.

Table 8

	Protease	Detergency index
		Detergent A
Detergent of the invention 1	<i>Bacillus sp.</i> KSM-KP43 (Example 2)	106
Detergent of the invention 2	<i>Bacillus sp.</i> KSM-KP1790 (Example 3)	106
Detergent of the invention 3	<i>Bacillus sp.</i> KSM-KP9860 (Example 3)	105
Comparative detergent 1	Savinase 120T type White® (Novo Nordisk)	103.5
Comparative detergent 2	Durazym 6. 0T® (Novo Nordisk)	103.5
Comparative detergent 3	None	100

[0088] Table 8 demonstrates that, even under the same activity conditions, the detergent composition containing the enzyme of the present invention (detergent A) exhibits superior detergency as compared to detergents containing conventional proteases. Detergents B and C also exhibit excellent detergency of the present invention.

Example 7

[0089] A granular product was prepared through a method disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 62-257990 by use of a purified sample of protease of the present invention which had been derived from *Bacillus sp.* KSM-KP43, KSM-KP1790, or KSM-KP9860 and prepared in Example 2 or 3. The granular product (6 APU/g) (1 part by weight) was incorporated into each of detergents (100 parts by weight) having compositions shown in Table 9, to thereby obtain detergent compositions of the present invention. Then the detergent was of the granular type, such a detergent was prepared by blending a granular detergent base which is free of components; i.e., an enzyme, PC, AC-1, and AC-2, with a granulated enzyme, granulated PC, granulated AC-1, and granulated AC-2. Each detergent was dissolved in water containing 71.2 mg CaCO₃/L (4° DH) at a concentration for use, and a collar was washed in a manner as described in Example 6. The detergents produced herein exhibit excellent washing power, and are useful for a laundry detergent.

Table 9

Component (%)	Detergents of the present invention									
	4	5	6	7	8	9	10	11	12	13
LAS-2	20		20.5		12				5	10
LAS-3		15								
AS-2			5		10		20			
SAS	3									
AOS		3								
SFE		8								
Fatty acid salt	2	6	4	10	3	3	2	1.5		
AES-2								20		
AE-3	3									10
AE-4		3	3	15		15	3		15	
AE-5							2	20	20	25
AG									5	7
Zeolite	30	18	15	15		10	20			
Oil-absorbing carrier				10		12				
Crystalline silicate				20						
Amorphous silicate	12	1	8		10	-	5			
STPP					25.5	20				
Sodium carbonate	10	27	25	10	10	15	17.5	0.1		
Potassium carbonate		3		2	5					
Sodium sulfite	2	.2			1			0.2	0.2	0.2
Sodium sulfate	4.5	1.5		1	11	8	10			
Sodium citrate			4	2			5	1.5	1	1
NTA						2				
Monoethanol amine								4	5	6
PAA					1	1.5	3			
AA-MA		3	3	5						
CMC	2									
PEG	5	2	2	2	2			1.5		
PVP							2			
Fluorescent dye	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.1	0.1	0.1
Perfume	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.3
Water	4	5	3	0.5	6	1	5	43.7	38.2	30.2
Ethanol								5	5	5
Propylene glycol								2	5	5
Enzyme	2	2	2	3	3	2	2	0.1	0.2	0.2

Component (%)	Detergents of the present invention									
	4	5	6	7	8	9	10	11	12	13
PC			3	3	10	3				
AC-1			2							
AC-2				1						
Total	100	100	100	100	100	100	100	100	100	100
Form	G*	G*	G*	G*	G*	G*	G*	L**	L**	L**
Concentration in use	20g/30L	20g/30L	20g/30L	20g/30L	20g/30L	20g/30L	20g/30L	20mL/30L	20mL/30L	20mL/30L

*) G stands for granular.

**) L stands for liquid.

LSA-2: alkylbenzene sulfonic acid (C10-C14 alkyl chain) which was neutralized with 48% NaOH

LSA-3: alkylbenzene sulfonic acid (C10-C14 alkyl chain) which was neutralized with 50% NaOH

AS-2: sodium salt of Dovanol 25 sulfate (C12-C15 sulfate)

SAS: sodium C13-C18 alkane sulfonate

AOS: sodium α -olefin sulfonate

SFE: sodium salt of palm oil α -sulfofatty acid methyl ester

Fatty acid salt: sodium palmitate

AES-2: sodium polyoxyethylene alkyl (C12-C15) ether sulfate (average EO addition of 2 moles)

AE-3: EO adduct (average 3 moles) of C12-C13 alcohol

AE-4: EO adduct (average 7.2 moles) of C12-C15 alcohol

AE-5: EO adduct (average 7 moles) of C12-C15 secondary alcohol

AG: alkyl (palm oil-derived) glucoside (average polymerization degree of 1.5)

Oil-absorbing carrier: Amorphous sodium aluminosilicate, oil absorption of 235 mL/100 g

Crystalline silicate: SKS-6 (δ -Na₂Si₂O₇, crystalline layered silicate, average particle size of 20 μ m)

Amorphous silicate: JIS No. 1 sodium silicate

STPP: sodium tripolyphosphate

NTA: sodium nitrilotriacetate

PAA: sodium salt of poly(acrylic acid), average molecular weight of 12,000

AA-MA: acrylic acid/maleic acid copolymer

CMC: carboxymethyl cellulose sodium

PEG: polyethylene glycol, average molecular weight of 6,000

PVA: polyvinylpyrrolidone, average molecular weight of 40,000.

K value of 26-35

Fluorescent dye: Tinopal CBS and Whitex SA (1 : 1 (wt.)),

only Cinoparl incorporated into a liquid detergent

Perfume: A perfume composition disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 8-239700

Enzyme: Lipolase 100T, Termamyl 60T, and KAC 500® (product of Kao Corporation) 1 : 1 : 1 (wt.)

PC: sodium percarbonate, average particle size of 400 μ m, coated with sodium metaborate

AC-1: tetraacetylenediamine

AC-2: sodium lauroxybenzene sulfonate

Example 8

[0090] Among the components shown in Table 10, sodium percarbonate and sodium carbonate (dense ash) were mixed with stirring. To the mixture, a 40% aqueous solution of sodium polyacrylate and sodium linear alkylbenzene sulfonate (or nonionic surfactant or sodium lauroxybenzene sulfonate) were added. Subsequently, a granulation product of alkaline protease which had been derived from *Bacillus* sp. KSM-KP43 and prepared in Example 7 was added to the mixture. The resultant mixture was homogeneously stirred, to thereby prepare a bleaching agent. A collar was immersed in a 0.5% aqueous solution of each of the bleaching agents at 20°C for 30 minutes, and subsequently washed with detergent A (Example 6) in a Terg-O-Tometer at 100 rpm for 10 minutes at 20°C. The obtained bleaching agents have excellent bleaching ability, and are useful as a bleaching agent for laundry.

Table 10

Component	(wt.%)			
	Bleaching agents of the present invention			
	14	15	16	17
Sodium percarbonate ¹⁾	80.0	80.0	80.0	80.0
Sodium carbonate (dense ash)	16.0	12.0	16.0	12.0
Anionic surfactant ²⁾	2.0	2.0	-	-
Nonionic surfactant ³⁾	-	-	2.0	2.0
Sodium polyacrylate ⁴⁾	1.0	1.0	1.0	1.0
Sodium lauroxybenzene sulfonate	-	4.0	-	4.0
<i>Bacillus</i> sp. KSM-KP43 Alkaline protease (Ex. 7)	1.0	1.0	1.0	1.0

1) Particle size: 500-700 μ m

2) Sodium linear alkylbenzene sulfonate (C12-C14)

3) Polyoxyethylene alkyl ether (C12-C14 alkyl, average EO addition of 12 mol)

4) Average molecular weight of 8,000

Example 9

[0091] The procedure of Example 8 was repeated, to thereby prepare detergent compositions for an automated dishwasher having a composition shown in Table 11. Washing power of the obtained compositions was tested under

the following conditions. The obtained detergents have excellent washing power, and are useful as a detergent for an automated dishwasher.

Table 11

Component	(wt. %)			
	Detergents of the present invention			
	18	19	20	21
Pluronic L-61 ¹⁾	4	-	4	4
Softanol EP-7085 ²⁾	-	4	-	-
Trisodium citrate	30	30	-	-
EDTA	-	-	30	-
Sodium tripolyphosphate	-	-	-	30
Sodium percarbonate	20	20	20	20
Sodium carbonate (dense ash)	20	20	20	20
Amorphous silicate ³⁾	10	10	10	10
AA-MA ⁴⁾	4	4	4	4
Sodium sulfate	10	10	10	10
Lipolase 100T [®] (Novo Nordisk)	0.5	0.5	0.5	0.5
Termamyl 60T [®] (Novo Nordisk)	1	1	1	1
<i>Bacillus</i> sp. KSM-KP43 alkaline protease (Ex. 7)	0.5	0.5	0.5	0.5

1) Polyoxyethylene-polyoxypropylene copolymer (average molecular weight of 2,000)

2) Ethylene oxide (7 moles) and propylene oxide (8.5 moles) adduct of C12-C14 sec-alcohol

3) JIS No. 2 sodium silicate

4) Acrylic acid-maleic acid copolymer

35 (1) Preparation of a soiled dish

[0092] Egg yolk (2.5 g) was homogeneously brushed onto one ceramic dish having a diameter of 25 cm. The dish was dried in a drier at 115°C for 60 minutes.

40 (2) Washing conditions

[0093]

Washer used; Full automated dishwasher (NP-810, product of Matsushita Electric Industry Co., Ltd.)

Type of washing; Standard course

Water for washing; Hardness of 62.3 mg CaCO₃/L (3.5° DH)

Concentration of detergent; 0.2 wt. %

50 (3) Method for evaluation

[0094] Five soiled dishes were washed in the washer under the above conditions by use of the detergent compositions of Example 9. The washed dish was stained with a 1% Erythrosine solution, to thereby color residual protein. The degree of protein soil was judged visually.

55 Example 10

[0095] Detergent compositions for an automated dishwasher were obtained from components shown in Table 12. Washing power of these compositions were evaluated through a test similar to that of Example 9. The compositions pro-

vided an excellent washing effect.

Table 12

		(wt.%)				
Component		Detergent compositions of the present invention				
		22	23	24	25	26
(a)	Sodium carbonate	30		30		50
	Sodium hydrogencarbonate		25		25	
(b)	Sokalan CP5 ¹⁾	5	6	5	5	5
(c)	Sodium hydrogenpercarbonate	5		6		
(d)	Limonene	2	2		1	1
	Softanol EP7045 ²⁾			2	1	1
(c)	Amorphous sodium aluminosilicate (Synth. Ex.1) ³⁾	2		2	1	3
	Amorphous sodium aluminosilicate (Synth. Ex.2) ⁴⁾		2		1	
	Lipolase 100T [®] (Novo Nordisk)	0.5	0.5	0.5	0.5	0.5
	Termamyl 60T [®] (Novo Nordisk)	1	1	1	1	1
	<i>Bacillus</i> sp. KSM-KP43 alkaline protease (Ex.7)	0.5	0.5	0.5	0.5	0.5
	Sodium malate		10		5	
	Sodium citrate	15		10	4	8
	Sodium sulfate	39	53	43	55	30

1) Acrylic acid/maleic acid copolymer (product of BASF)

2) Ethylene oxide (7 moles) and propylene oxide (4.5 moles) adduct of C12-C14 sec-alcohol

3), 4) Synthetic Example disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 6-179899

Example 11

[0096] Enzymes were added to the above-described detergent A (Example 6) in amounts shown in the following Table 13. A collar portion of a white shirt was washed in a manner similar to that of Example 6.

Table 13

		(wt.%)						
Enzyme		Detergents of the present invention						
		27	28	29	30	31	32	33
	Protease of the present invention ¹⁾	-	0.5	0.5	0.5	0.5	0.5	0.5
	Conventional protease ²⁾	-	-	0.6	-	-	0.6	0.6
	Cellulase ³⁾	-	-	-	0.7	-	0.7	0.7
	Lipase ⁴⁾	-	-	-	-	0.5	-	0.5

1) A granular product prepared through a method disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 62-257990 by use of a purified sample of protease of the present invention which was derived from *Bacillus* sp. KSM-KP 43 strain and prepared in Example 2 (6 APU/g)

2) Protease K-16 disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 5-25492 which was modified to have 5 APU/g through a method disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 62-257990

3) KAC-500[®] (cellulase, 500 U/g, product of Kao Corporation)

4) Lipolase 100T[®] (product of Novo Nordisk)

[0097] The results clearly show that the combination of the protease of the present invention and a conventional protease, cellulase, or lipase enhances a washing effect.

Industrial Applicability

5

[0098] The alkaline protease of the present invention has excellent stability against a variety of surfactants; resistance to fatty acids; and high stability against an oxidizing agent, and is therefore useful as an enzyme for a detergent for an automated dishwasher and for a laundry detergent, both containing a bleaching component.

10

15

20

25

30

35

40

45

50

55

SEQUENCE LISTING

5 <110> KAO CORPORATION

10 <120> Alkaline Protease

15 <130> FP-KS-0498

<150> JP 09-274570

20 <151> 1997-10-07

25 <160> 5

<210> 1

30 <211> 639

<212> PRT

35 <213> *Bacillus sp.*

<220>

40 <221> misc_feature

45 <222> 23. 29. 32. 46. 47. 53. 70. 74. 89. 102. 105. 128. 130. 131. 132. 133. 146.
148. 160. 165. 172. 183. 187. 188. 189. 194. 286. 306. 324. 369. 431. 501.
531. 541. 584. 591. 592. 594. 595. 596. 611. 632

50 <223> Xaa=arbitraty amino acid

55 <400>

	Met	Arg	Lys	Lys	Lys	Val	Phe	Leu	Ser	Val	Leu	Ser	Ala	Ala	Ala	Ile
5	1			5					10					15		
	Leu	Ser	Thr	Val	Ala	Leu	Xaa	Asn	Pro	Ser	Ala	Gly	Xaa	Ala	Arg	Xaa
				20					25					30		
10	Phe	Asp	Leu	Asp	Phe	Lys	Gly	Ile	Gln	Thr	Thr	Thr	Asp	Xaa	Xaa	Gly
				35					40					45		
15	Phe	Ser	Lys	Gln	Xaa	Gln	Thr	Gly	Ala	Ala	Ala	Phe	Leu	Val	Glu	Ser
				50					55					60		
	Glu	Asn	Val	Lys	Leu	Xaa	Lys	Gly	Leu	Xaa	Lys	Lys	Leu	Glu	Thr	Val
20				65					70					75		80
	Pro	Ala	Asn	Asn	Lys	Leu	His	Ile	Xaa	Gln	Phe	Asn	Gly	Pro	Ile	Leu
25					85					90					95	
	Glu	Glu	Thr	Lys	Gln	Xaa	Leu	Glu	Xaa	Thr	Gly	Ala	Lys	Ile	Leu	Asp
				100					105					110		
30	Tyr	Ile	Pro	Asp	Tyr	Ala	Tyr	Ile	Val	Glu	Tyr	Glu	Gly	Asp	Val	Xaa
				115					120					125		
35	Ser	Xaa	Xaa	Xaa	Xaa	Ile	Glu	His	Val	Glu	Ser	Val	Glu	Pro	Tyr	Leu
				130					135					140		
	Pro	Xaa	Tyr	Xaa	Ile	Asp	Pro	Gln	Leu	Phe	Thr	Lys	Gly	Ala	Ser	Xaa
40				145					150					155		160
	Leu	Val	Lys	Ala	Xaa	Ala	Leu	Asp	Thr	Lys	Gln	Xaa	Asn	Lys	Glu	Val
45					165					170					175	
	Gln	Leu	Arg	Gly	Ile	Glu	Xaa	Ile	Ala	Gln	Xaa	Xaa	Xaa	Ser	Asn	Asp
				180						185					190	
50	Val	Xaa	Tyr	Ile	Thr	Ala	Lys	Pro	Glu	Tyr	Lys	Val	Met	Asn	Asp	Val
				195						200					205	
55																

5 Ala Arg Gly Ile Val Lys Ala Asp Val Ala Gln Ser Ser Tyr Gly Leu
 210 215 220
 Tyr Gly Gln Gly Gln Ile Val Ala Val Ala Asp Thr Gly Leu Asp Thr
 225 230 235 240
 10 Gly Arg Asn Asp Ser Ser Met His Glu Ala Phe Arg Gly Lys Ile Thr
 245 250 255
 15 Ala Leu Tyr Ala Leu Gly Arg Thr Asn Asn Ala Asn Asp Thr Asn Gly
 260 265 270
 His Gly Thr His Val Ala Gly Ser Val Leu Gly Asn Gly Xaa Thr Asn
 20 275 280 285
 Lys Gly Met Ala Pro Gln Ala Asn Leu Val Phe Gln Ser Ile Met Asp
 290 295 300
 25 Ser Xaa Gly Gly Leu Gly Gly Leu Pro Ser Asn Leu Gln Thr Leu Phe
 305 310 315 320
 30 Ser Gln Ala Xaa Ser Ala Gly Ala Arg Ile His Thr Asn Ser Trp Gly
 325 330 335
 Ala Ala Val Asn Gly Ala Tyr Thr Thr Asp Ser Arg Asn Val Asp Asp
 35 340 345 350
 Tyr Val Arg Lys Asn Asp Met Thr Ile Leu Phe Ala Ala Gly Asn Glu
 40 355 360 365
 Xaa Pro Asn Gly Gly Thr Ile Ser Ala Pro Gly Thr Ala Lys Asn Ala
 370 375 380
 45 Ile Thr Val Gly Ala Thr Glu Asn Leu Arg Pro Ser Phe Gly Ser Tyr
 385 390 395 400
 50 Ala Asp Asn Ile Asn His Val Ala Gln Phe Ser Ser Arg Gly Pro Thr
 405 410 415
 55

Lys Asp Gly Arg Ile Lys Pro Asp Val Met Ala Pro Gly Thr Xaa Ile
 5 420 425 430
 Leu Ser Ala Arg Ser Ser Leu Ala Pro Asp Ser Ser Phe Trp Ala Asn
 435 440 445
 10 His Asp Ser Lys Tyr Ala Tyr Met Gly Gly Thr Ser Met Ala Thr Pro
 450 455 460
 15 Ile Val Ala Gly Asn Val Ala Gln Leu Arg Glu His Phe Val Lys Asn
 465 470 475 480
 Arg Gly Ile Thr Pro Lys Pro Ser Leu Leu Lys Ala Ala Leu Ile Ala
 20 485 490 495
 Gly Ala Ala Asp Xaa Gly Leu Gly Tyr Pro Asn Gly Asn Gln Gly Trp
 25 500 505 510
 Gly Arg Val Thr Leu Asp Lys Ser Leu Asn Val Ala Tyr Val Asn Glu
 515 520 525
 30 Ser Ser Xaa Leu Ser Thr Ser Gln Lys Ala Thr Tyr Xaa Phe Thr Ala
 530 535 540
 35 Thr Ala Gly Lys Pro Leu Lys Ile Ser Leu Val Trp Ser Asp Ala Pro
 545 550 555 560
 Ala Ser Thr Thr Ala Ser Val Thr Leu Val Asn Asp Leu Asp Leu Val
 40 565 570 575
 Ile Thr Ala Pro Asn Gly Thr Xaa Tyr Val Gly Asn Asp Phe Xaa Xaa
 45 580 585 590
 Pro Xaa Xaa Xaa Asn Trp Asp Gly Arg Asn Asn Val Glu Asn Val Phe
 595 600 605
 50 Ile Asn Xaa Pro Gln Ser Gly Thr Tyr Thr Ile Glu Val Gln Ala Tyr
 610 615 620
 55

Asn Val Pro Val Gly Pro Gln Xaa Phe Ser Leu Ala Ile Val Asn
 5 625 630 635
 <210> 2
 <211> 640
 10 <212> PRT
 <213> *Bacillus* sp.
 15
 <220>
 <221> misc_feature
 20 <222> 3, 24, 30, 33, 47, 48, 54, 71, 75, 90, 103, 106, 129, 131, 132, 133, 134, 147,
 149, 161, 166, 173, 184, 188, 189, 190, 195, 287, 307, 325, 370, 432, 502,
 25 532, 542, 585, 592, 593, 595, 596, 597, 612, 633
 <223> Xaa=arbitrary amino acid
 30
 <400>
 Met Arg Xaa Lys Lys Lys Val Phe Leu Ser Val Leu Ser Ala Ala Ala
 35 1 5 10 15
 Ile Leu Ser Thr Val Ala Leu Xaa Asn Pro Ser Ala Gly Xaa Ala Arg
 20 25 30
 40 Xaa Phe Asp Leu Asp Phe Lys Gly Ile Gln Thr Thr Thr Asp Xaa Xaa
 35 40 45
 Gly Phe Ser Lys Gln Xaa Gln Thr Gly Ala Ala Ala Phe Leu Val Glu
 45 50 55 60
 Ser Glu Asn Val Lys Leu Xaa Lys Gly Leu Xaa Lys Lys Leu Glu Thr
 50 65 70 75 80
 Val Pro Ala Asn Asn Lys Leu His Ile Xaa Gln Phe Asn Gly Pro Ile

55

	85	90	95
5	Leu Glu Glu Thr Lys Gln Xaa	Leu Glu Xaa Thr Gly Ala Lys	Ile Leu
	100	105	110
10	Asp Tyr Ile Pro Asp Tyr Ala Tyr Ile Val Glu Tyr Glu Gly Asp Val		
	115	120	125
15	Xaa Ser Xaa Xaa Xaa Xaa Ile Glu His Val Glu Ser Val Glu Pro Tyr		
	130	135	140
20	Leu Pro Xaa Tyr Xaa Ile Asp Pro Gln Leu Phe Thr Lys Gly Ala Ser		
	145	150	155
25	Xaa Leu Val Lys Ala Xaa Ala Leu Asp Thr Lys Gln Xaa Asn Lys Glu		
	165	170	175
30	Val Gln Leu Arg Gly Ile Glu Xaa Ile Ala Gln Xaa Xaa Xaa Ser Asn		
	180	185	190
35	Asp Val Xaa Tyr Ile Thr Ala Lys Pro Glu Tyr Lys Val Met Asn Asp		
	195	200	205
40	Val Ala Arg Gly Ile Val Lys Ala Asp Val Ala Gln Ser Ser Tyr Gly		
	210	215	220
45	Leu Tyr Gly Gln Gly Gln Ile Val Ala Val Ala Asp Thr Gly Leu Asp		
	225	230	235
50	Thr Gly Arg Asn Asp Ser Ser Met His Glu Ala Phe Arg Gly Lys Ile		
	245	250	255
55	Thr Ala Leu Tyr Ala Leu Gly Arg Thr Asn Asn Ala Asn Asp Thr Asn		
	260	265	270
	Gly His Gly Thr His Val Ala Gly Ser Val Leu Gly Asn Gly Xaa Thr		
	275	280	285
	Asn Lys Gly Met Ala Pro Gln Ala Asn Leu Val Phe Gln Ser Ile Met		

EP 1 029 920 A1

	290	295	300	
5	Asp Ser Xaa Gly Gly Leu Gly Gly Leu Pro Ser Asn Leu Gln Thr Leu			
	305	310	315	320
10	Phe Ser Gln Ala Xaa Ser Ala Gly Ala Arg Ile His Thr Asn Ser Trp			
		325	330	335
15	Gly Ala Ala Val Asn Gly Ala Tyr Thr Thr Asp Ser Arg Asn Val Asp			
	340	345	350	
20	Asp Tyr Val Arg Lys Asn Asp Met Thr Ile Leu Phe Ala Ala Gly Asn			
	355	360	365	
25	Glu Xaa Pro Asn Gly Gly Thr Ile Ser Ala Pro Gly Thr Ala Lys Asn			
	370	375	380	
30	Ala Ile Thr Val Gly Ala Thr Glu Asn Leu Arg Pro Ser Phe Gly Ser			
	385	390	395	400
35	Tyr Ala Asp Asn Ile Asn His Val Ala Gln Phe Ser Ser Arg Gly Pro			
	405	410	415	
40	Thr Lys Asp Gly Arg Ile Lys Pro Asp Val Met Ala Pro Gly Thr Xaa			
	420	425	430	
45	Ile Leu Ser Ala Arg Ser Ser Leu Ala Pro Asp Ser Ser Phe Trp Ala			
	435	440	445	
50	Asn His Asp Ser Lys Tyr Ala Tyr Met Gly Gly Thr Ser Met Ala Thr			
	450	455	460	
55	Pro Ile Val Ala Gly Asn Val Ala Gln Leu Arg Glu His Phe Val Lys			
	465	470	475	480
	Asn Arg Gly Ile Thr Pro Lys Pro Ser Leu Leu Lys Ala Ala Leu Ile			
	485	490	495	
	Ala Gly Ala Ala Asp Xaa Gly Leu Gly Tyr Pro Asn Gly Asn Gln Gly			

500 505 510
 5 Trp Gly Arg Val Thr Leu Asp Lys Ser Leu Asn Val Ala Tyr Val Asn
 515 520 525
 10 Glu Ser Ser Xaa Leu Ser Thr Ser Gln Lys Ala Thr Tyr Xaa Phe Thr
 530 535 540
 Ala Thr Ala Gly Lys Pro Leu Lys Ile Ser Leu Val Trp Ser Asp Ala
 15 545 550 555 560
 Pro Ala Ser Thr Thr Ala Ser Val Thr Leu Val Asn Asp Leu Asp Leu
 565 570 575
 20 Val Ile Thr Ala Pro Asn Gly Thr Xaa Tyr Val Gly Asn Asp Phe Xaa
 580 585 590
 25 Xaa Pro Xaa Xaa Xaa Asn Trp Asp Gly Arg Asn Asn Val Glu Asn Val
 595 600 605
 Phe Ile Asn Xaa Pro Gln Ser Gly Thr Tyr Thr Ile Glu Val Gln Ala
 30 610 615 620
 Tyr Asn Val Pro Val Gly Pro Gln Xaa Phe Ser Leu Ala Ile Val Asn
 35 625 630 635 640

 <210> 3
 40 <211> 1920
 <212> DNA
 45 <213> *Bacillus sp.*

 <400>
 50 atg aga aag aag aag gtg ttt tta tct gtt tta tca gct gca gcg att 48
 Met Arg Lys Lys Lys Val Phe Leu Ser Val Leu Ser Ala Ala Ala Ile
 55

	1	5	10	15	
5	ctg	tcg act gtt gca tta aac aat ccc tcg gct ggt gat gca agg act	96		
	Leu Ser Thr Val Ala Leu Asn Asn Pro Ser Ala Gly Asp Ala Arg Thr				
	20	25	30		
10	ttt gat ctg gat ttt aaa gga att caa aca aca acc gat gtc agt ggt	144			
	Phe Asp Leu Asp Phe Lys Gly Ile Gln Thr Thr Thr Asp Val Ser Gly				
15	35	40	45		
	ttc tcc aaa cag cga caa aca ggt gcg gct gca ttt ctg gtg gag tct	192			
	Phe Ser Lys Gln Arg Gln Thr Gly Ala Ala Ala Phe Leu Val Glu Ser				
20	50	55	60		
	gaa aat gtg aaa ctt ctt aaa gga ttg cta aag aaa ctt gaa aca gta	240			
25	Glu Asn Val Lys Leu Leu Lys Gly Leu Leu Lys Lys Leu Glu Thr Val				
	65	70	75	80	
	ccg gca aat aat aaa ctc cat att gtc caa ttc aat ggc ccc att tta	288			
30	Pro Ala Asn Asn Lys Leu His Ile Val Gln Phe Asn Gly Pro Ile Leu				
	85	90	95		
35	gaa gaa aca aaa cag aag cta gag aca act gga gca aag att ctc gac	336			
	Glu Glu Thr Lys Gln Lys Leu Glu Thr Thr Gly Ala Lys Ile Leu Asp				
	100	105	110		
40	tac atc cct gat tat gca tat att gtc gag tat gag ggg gat gtt cag	384			
	Tyr Ile Pro Asp Tyr Ala Tyr Ile Val Glu Tyr Glu Gly Asp Val Gln				
45	115	120	125		
	tca aaa gtc cgc tcc att gaa cac gtg gaa tca gtg gag cca tac ttg	432			
	Ser Lys Val Arg Ser Ile Glu His Val Glu Ser Val Glu Pro Tyr Leu				
50	130	135	140		
	ccg aaa tac aaa ata gat ccc cag ctt ttc aca aaa ggc gca tcg acg	480			

55

Pro Lys Tyr Lys Ile Asp Pro Gln Leu Phe Thr Lys Gly Ala Ser Thr
 5 145 150 155 160
 ctg gtg aaa gcg ttg gcg ctt gat acg aag cag aac aat aaa gaa gtg 528
 Leu Val Lys Ala Leu Ala Leu Asp Thr Lys Gln Asn Asn Lys Glu Val
 10 165 170 175
 caa tta aga ggc atc gag gaa atc gct cag tac gta gca agc aat gac 576
 15 Gln Leu Arg Gly Ile Glu Glu Ile Ala Gln Tyr Val Ala Ser Asn Asp
 180 185 190
 gtc cat tat att acg gca aag cct gaa tat aag gtg atg aat gat gtg 624
 20 Val His Tyr Ile Thr Ala Lys Pro Glu Tyr Lys Val Met Asn Asp Val
 195 200 205
 gcc aga ggt att gtc aaa gcg gat gtg gca cag agc agc tac ggt ttg 672
 25 Ala Arg Gly Ile Val Lys Ala Asp Val Ala Gln Ser Ser Tyr Gly Leu
 210 215 220
 30 tat gga caa ggc cag att gtc gca gtt gcc gat act gga ttg gat aca 720
 Tyr Gly Gln Gly Gln Ile Val Ala Val Ala Asp Thr Gly Leu Asp Thr
 35 225 230 235 240
 gga aga aac gac agt tcg atg cat gaa gcc ttc cgc ggt aaa ata aca 768
 Gly Arg Asn Asp Ser Ser Met His Glu Ala Phe Arg Gly Lys Ile Thr
 40 245 250 255
 gca cta tat gca ctg ggt cgg acg aat aat gcg aat gat acg aac ggt 816
 45 Ala Leu Tyr Ala Leu Gly Arg Thr Asn Asn Ala Asn Asp Thr Asn Gly
 260 265 270
 cat ggt acc cat gtg gca ggt tcg gta tta gga aat ggc gca acg aat 864
 50 His Gly Thr His Val Ala Gly Ser Val Leu Gly Asn Gly Ala Thr Asn
 275 280 285
 55

5 aaa gga atg gca cct caa gcg aat ctg gtt ttt caa tcc atc atg gat 912
 Lys Gly Met Ala Pro Gln Ala Asn Leu Val Phe Gln Ser Ile Met Asp
 290 295 300
 10 agc agt ggt ggg ctt gga ggc ttg cct tcc aat ctg caa acc tta ttc 960
 Ser Ser Gly Gly Leu Gly Gly Leu Pro Ser Asn Leu Gln Thr Leu Phe
 305 310 315 320
 15 agc caa gca ttc agt gca ggt gcc aga att cat aca aac tcc tgg ggg 1008
 Ser Gln Ala Phe Ser Ala Gly Ala Arg Ile His Thr Asn Ser Trp Gly
 325 330 335
 20 gca gcg gtg aat ggg gcc tac acg aca gat tcc aga aat gtg gat gac 1056
 Ala Ala Val Asn Gly Ala Tyr Thr Thr Asp Ser Arg Asn Val Asp Asp
 340 345 350
 25 tat gta agg aaa aat gat atg acg att ctt ttc gcg gct ggg aat gaa 1104
 Tyr Val Arg Lys Asn Asp Met Thr Ile Leu Phe Ala Ala Gly Asn Glu
 355 360 365
 30 agg ccg aac ggc ggt acc atc agt gca cct ggt acg gct aaa aac gcc 1152
 Arg Pro Asn Gly Gly Thr Ile Ser Ala Pro Gly Thr Ala Lys Asn Ala
 370 375 380
 40 ata aca gtc ggc gca acc gaa aac ctg cgt cca agc ttc ggt tcc tat 1200
 Ile Thr Val Gly Ala Thr Glu Asn Leu Arg Pro Ser Phe Gly Ser Tyr
 385 390 395 400
 45 gca gat aat att aac cac gtt gca cag ttc tct tcc cgt ggc ccg aca 1248
 Ala Asp Asn Ile Asn His Val Ala Gln Phe Ser Ser Arg Gly Pro Thr
 405 410 415
 50 aaa gat ggg cga atc aag cct gat gtc atg gcg cca ggg aca tac att 1296
 Lys Asp Gly Arg Ile Lys Pro Asp Val Met Ala Pro Gly Thr Tyr Ile
 55

5 420 425 430
 tta tca gca aga tct tct ctt gca ccc gat tcc tcc ttc tgg gcg aat 1344
 Leu Ser Ala Arg Ser Ser Leu Ala Pro Asp Ser Ser Phe Trp Ala Asn
 10 435 440 445
 cat gac agc aaa tat gcc tat atg ggt gga acg tcc atg gca aca ccg 1392
 His Asp Ser Lys Tyr Ala Tyr Met Gly Gly Thr Ser Met Ala Thr Pro
 15 450 455 460
 att gtt gcg ggg aat gtt gca cag ctg cgt gag cat ttt gtg aaa aat 1440
 Ile Val Ala Gly Asn Val Ala Gln Leu Arg Glu His Phe Val Lys Asn
 20 465 470 475 480
 aga gga atc act cct aag cct tcc cta ttg aaa gca gct ttg att gca 1488
 Arg Gly Ile Thr Pro Lys Pro Ser Leu Leu Lys Ala Ala Leu Ile Ala
 25 485 490 495
 ggt gct gct gat gtt gga ttg ggt tat ccg aac gga aac caa gga tgg 1536
 Gly Ala Ala Asp Val Gly Leu Gly Tyr Pro Asn Gly Asn Gln Gly Trp
 30 500 505 510
 ggc cga gtg acc ctg gat aaa tcg ttg aac gtt gcc tat gtg aac gaa 1584
 Gly Arg Val Thr Leu Asp Lys Ser Leu Asn Val Ala Tyr Val Asn Glu
 35 515 520 525
 tcc agt gcc cta tca act agc caa aaa gcg aca tat acc ttt act gca 1632
 Ser Ser Ala Leu Ser Thr Ser Gln Lys Ala Thr Tyr Thr Phe Thr Ala
 40 530 535 540
 acg gcg ggc aag cca ttg aaa atc tcc ctg gta tgg tcg gat gcc cct 1680
 Thr Ala Gly Lys Pro Leu Lys Ile Ser Leu Val Trp Ser Asp Ala Pro
 45 545 550 555 560
 gca agc act act gct tct gta acc ctg gtc aat gat ttg gat ttg gtc 1728
 55

Ala Ser Thr Thr Ala Ser Val Thr Leu Val Asn Asp Leu Asp Leu Val
5 565 570 575
att aca gca cca aac gga aca aga tat gtc ggg aat gac ttc tca gca 1776
Ile Thr Ala Pro Asn Gly Thr Arg Tyr Val Gly Asn Asp Phe Ser Ala
10 580 585 590
cca ttt gac aat aac tgg gat ggc cgc aat aac gta gaa aat gta ttt 1824
15 Pro Phe Asp Asn Asn Trp Asp Gly Arg Asn Asn Val Glu Asn Val Phe
595 600 605
att aat tcg ccc caa agt gga aca tat acc att gag gtg caa gca tat 1872
20 Ile Asn Ser Pro Gln Ser Gly Thr Tyr Thr Ile Glu Val Gln Ala Tyr
610 615 620
25 aat gtg ccg gtt gga cca caa aac ttc tcg ttg gca att gtg aac taa 1920
Asn Val Pro Val Gly Pro Gln Asn Phe Ser Leu Ala Ile Val Asn
625 630 635
30
<210> 4
35 <211> 1923
<212> DNA
40 <213> *Bacillus sp.*
<400>
45 atg aga aag aag aaa aag gtg ttt tta tct gtt tta tca gct gca gcg 48
Met Arg Lys Lys Lys Lys Val Phe Leu Ser Val Leu Ser Ala Ala Ala
1 5 10 15
50 att ttg tcg act gtt gcg tta agt aat cca tct gca ggt ggt gca agg 96
Ile Leu Ser Thr Val Ala Leu Ser Asn Pro Ser Ala Gly Gly Ala Arg
55

	20	25	30	
5	aat ttt gat ctg gat ttc aaa gga att cag aca aca act gat gct aaa 144			
	Asn Phe Asp Leu Asp Phe Lys Gly Ile Gln Thr Thr Thr Asp Ala Lys			
	35	40	45	
10	ggt ttc tcc aag cag ggg cag act ggt gct gct gct ttt ctg gtg gaa 192			
	Gly Phe Ser Lys Gln Gly Gln Thr Gly Ala Ala Ala Phe Leu Val Glu			
15	50	55	60	
	tct gaa aat gtg aaa ctc cca aaa ggt ttg cag aag aag ctt gaa aca 240			
	Ser Glu Asn Val Lys Leu Pro Lys Gly Leu Gln Lys Lys Leu Glu Thr			
20	65	70	75	80
	gtc ccg gca aat aat aaa ctc cat att atc caa ttc aat gga cca att 288			
25	Val Pro Ala Asn Asn Lys Leu His Ile Ile Gln Phe Asn Gly Pro Ile			
	85	90	95	
	tta gaa gaa aca aaa cag cag ctg gaa aaa aca ggg gca aag att ctc 336			
30	Leu Glu Glu Thr Lys Gln Gln Leu Glu Lys Thr Gly Ala Lys Ile Leu			
	100	105	110	
35	gac tac ata cct gat tat gct tac att gtc gag tat gag ggc gat gtt 384			
	Asp Tyr Ile Pro Asp Tyr Ala Tyr Ile Val Glu Tyr Glu Gly Asp Val			
	115	120	125	
40	aag tca gca aca agc acc att gag cac gtg gaa tcc gtg gag cct tat 432			
	Lys Ser Ala Thr Ser Thr Ile Glu His Val Glu Ser Val Glu Pro Tyr			
45	130	135	140	
	ttg ccg ata tac aga ata gat ccc cag ctt ttc aca aaa ggg gca tca 480			
	Leu Pro Ile Tyr Arg Ile Asp Pro Gln Leu Phe Thr Lys Gly Ala Ser			
50	145	150	155	160
	gag ctt gta aaa gca gtg gcg ctt gat aca aag cag aaa aat aaa gag 528			

55

55

gat agc ggt ggg gga ctt gga gga cta cct tcg aat ctg caa acc tta 960
 5 Asp Ser Gly Gly Gly Leu Gly Gly Leu Pro Ser Asn Leu Gln Thr Leu
 305 310 315 320
 ttc agc caa gca tac agt gct ggt gcc aga att cat aca aac tcc tgg 1008
 10 Phe Ser Gln Ala Tyr Ser Ala Gly Ala Arg Ile His Thr Asn Ser Trp
 325 330 335
 gga gca gca gtg aat ggg gct tac aca aca gat tcc aga aat gtg gat 1056
 15 Gly Ala Ala Val Asn Gly Ala Tyr Thr Thr Asp Ser Arg Asn Val Asp
 340 345 350
 gac tat gtg cgc aaa aat gat atg acg atc ctt ttc gct gcc ggg aat 1104
 20 Asp Tyr Val Arg Lys Asn Asp Met Thr Ile Leu Phe Ala Ala Gly Asn
 355 360 365
 gaa gga ccg aac ggc gga acc atc agt gca cca ggc aca gct aaa aat 1152
 25 Glu Gly Pro Asn Gly Gly Thr Ile Ser Ala Pro Gly Thr Ala Lys Asn
 370 375 380
 gca ata aca gtc gga gct acg gaa aac ctc cgc cca agc ttt ggg tct 1200
 35 Ala Ile Thr Val Gly Ala Thr Glu Asn Leu Arg Pro Ser Phe Gly Ser
 385 390 395 400
 tat gcg gac aat atc aac cat gtg gca cag ttc tct tca cgt gga ccg 1248
 40 Tyr Ala Asp Asn Ile Asn His Val Ala Gln Phe Ser Ser Arg Gly Pro
 405 410 415
 aca aag gat gga cgg atc aaa ccg gat gtc atg gca ccg gga acg ttc 1296
 45 Thr Lys Asp Gly Arg Ile Lys Pro Asp Val Met Ala Pro Gly Thr Phe
 420 425 430
 ata cta tca gca aga tct tct ctt gca ccg gat tcc tcc ttc tgg gcg 1344
 50 Ile Leu Ser Ala Arg Ser Ser Leu Ala Pro Asp Ser Ser Phe Trp Ala
 55

5 435 440 445
 aac cat gac agt aaa tat gca tac atg ggt gga acg tcc atg gct aca 1392
 Asn His Asp Ser Lys Tyr Ala Tyr Met Gly Gly Thr Ser Met Ala Thr
 10 450 455 460
 ccg atc gtt gct gga aac gtg gca cag ctt cgt gag cat ttt gtg aaa 1440
 Pro Ile Val Ala Gly Asn Val Ala Gln Leu Arg Glu His Phe Val Lys
 15 465 470 475 480
 aac aga ggc atc aca cca aag cct tct cta tta aaa gcg gca ctg att 1488
 Asn Arg Gly Ile Thr Pro Lys Pro Ser Leu Leu Lys Ala Ala Leu Ile
 20 485 490 495
 gcc ggt gca gct gac atc ggc ctt ggc tac ccg aac ggt aac caa gga 1536
 Ala Gly Ala Ala Asp Ile Gly Leu Gly Tyr Pro Asn Gly Asn Gln Gly
 25 500 505 510
 tgg gga cga gtg aca ttg gat aaa tcc ctg aac gtt gcc tat gtg aac 1584
 Trp Gly Arg Val Thr Leu Asp Lys Ser Leu Asn Val Ala Tyr Val Asn
 30 515 520 525
 gag tcc agt tct cta tcc acc agc caa aaa gcg acg tac tcg ttt act 1632
 Glu Ser Ser Ser Leu Ser Thr Ser Gln Lys Ala Thr Tyr Ser Phe Thr
 35 530 535 540
 gct act gcc ggc aag cct ttg aaa atc tcc ctg gta tgg tct gat gcc 1680
 Ala Thr Ala Gly Lys Pro Leu Lys Ile Ser Leu Val Trp Ser Asp Ala
 40 545 550 555 560
 cct gcg agc aca act gct tcc gta acg ctt gtc aat gat ctg gac ctt 1728
 Pro Ala Ser Thr Thr Ala Ser Val Thr Leu Val Asn Asp Leu Asp Leu
 45 565 570 575
 gtc att acc gct cca aat ggc aca cag tat gta gga aat gac ttt act 1776
 50
 55

Val Ile Thr Ala Pro Asn Gly Thr Gln Tyr Val Gly Asn Asp Phe Thr
 5 580 585 590
 tcg cca tac aat gat aac tgg gat ggc cgc aat aac gta gaa aat gta 1824
 Ser Pro Tyr Asn Asp Asn Trp Asp Gly Arg Asn Asn Val Glu Asn Val
 10 595 600 605
 ttt att aat gca cca caa agc ggg acg tat aca att gag gta cag gct 1872
 Phe Ile Asn Ala Pro Gln Ser Gly Thr Tyr Thr Ile Glu Val Gln Ala
 15 610 615 620
 tat aac gta ccg gtt gga cca cag acc ttc tcg ttg gca att gtg aat 1920
 Tyr Asn Val Pro Val Gly Pro Gln Thr Phe Ser Leu Ala Ile Val Asn
 20 625 630 635 640
 taa 1923
 25
 <210> 5
 30 <211> 1923
 <212> DNA
 35 <212> *Bacillus sp.*
 <400>
 40 atg aga aag aag aaa aag gtg ttt tta tct gtt tta tca gct gca gcg 48
 Met Arg Lys Lys Lys Lys Val Phe Leu Ser Val Leu Ser Ala Ala Ala
 5 10 15
 45 att ttg tcg act gtt gcg tta agt aat cca tct gca ggt ggt gca agg 96
 Ile Leu Ser Thr Val Ala Leu Ser Asn Pro Ser Ala Gly Gly Ala Arg
 50 20 25 30
 aat ttt gat ctg gat ttc aaa gga att cag aca aca act gat gct aaa 144
 55

	Asn Phe Asp Leu Asp Phe Lys Gly Ile Gln Thr Thr Thr Asp Ala Lys	
5	35	40
	45	
	ggg ttc tcc aag cag ggg cag act ggt gct gct gct ttt ctg gtg gaa	192
10	Gly Phe Ser Lys Gln Gly Gln Thr Gly Ala Ala Ala Phe Leu Val Glu	
	50	55
	60	
	tct gaa aat gtg aaa ctc cca aaa ggt ttg cag aag aag ctt gaa aca	240
15	Ser Glu Asn Val Lys Leu Pro Lys Gly Leu Gln Lys Lys Leu Glu Thr	
	65	70
	75	80
	gtc ccg gca aat aat aaa ctc cat att atc caa ttc aat gga cca att	288
20	Val Pro Ala Asn Asn Lys Leu His Ile Ile Gln Phe Asn Gly Pro Ile	
	85	90
	95	
25	tta gaa gaa aca aaa cag cag ctg gaa aaa aca ggg gca aag att ctc	336
	Leu Glu Glu Thr Lys Gln Gln Leu Glu Lys Thr Gly Ala Lys Ile Leu	
	100	105
	110	
30	gac tac ata cct gat tat gct tac att gtc gag tat gag ggc gat gtt	384
	Asp Tyr Ile Pro Asp Tyr Ala Tyr Ile Val Glu Tyr Glu Gly Asp Val	
35	115	120
	125	
	aag tca gca aca agc acc att gag cac gtg gaa tcc gtg gag cct tat	432
40	Lys Ser Ala Thr Ser Thr Ile Glu His Val Glu Ser Val Glu Pro Tyr	
	130	135
	140	
	ttg ccg ata tac aga ata gat ccc cag ctt ttc aca aaa ggg gca tca	480
45	Leu Pro Ile Tyr Arg Ile Asp Pro Gln Leu Phe Thr Lys Gly Ala Ser	
	145	150
	155	160
	gag ctt gta aaa gca gtg gcg ctt gat aca aag cag aaa aat aaa gag	528
50	Glu Leu Val Lys Ala Val Ala Leu Asp Thr Lys Gln Lys Asn Lys Glu	
	165	170
	175	

5 gtg caa tta aga ggc atc gaa caa atc gca caa ttc gca ata agc aat 576
 Val Gln Leu Arg Gly Ile Glu Gln Ile Ala Gln Phe Ala Ile Ser Asn
 180 185 190
 10 gat gtg cta tat att acg gca aag cct gag tat aag gtg atg aat gat 624
 Asp Val Leu Tyr Ile Thr Ala Lys Pro Glu Tyr Lys Val Met Asn Asp
 195 200 205
 15 gtt gcg cgt gga att gtc aaa gcg gat gtg gct cag agc agc tac ggg 672
 Val Ala Arg Gly Ile Val Lys Ala Asp Val Ala Gln Ser Ser Tyr Gly
 210 215 220
 20 ttg tat gga caa gga cag atc gta gcg gtt gcc gat aca ggg ctt gat 720
 Leu Tyr Gly Gln Gly Gln Ile Val Ala Val Ala Asp Thr Gly Leu Asp
 25 225 230 235 240
 aca ggt cgc aat gac agt tcg atg cat gaa gcc ttc cgc ggg aaa att 768
 Thr Gly Arg Asn Asp Ser Ser Met His Glu Ala Phe Arg Gly Lys Ile
 30 245 250 255
 act gca tta tat gca ttg gga cgg acg aat aat gcc aat gat acg aat 816
 35 Thr Ala Leu Tyr Ala Leu Gly Arg Thr Asn Asn Ala Asn Asp Thr Asn
 260 265 270
 40 ggc cat ggt acg cat gtg gct ggc tcc gta tta gga aac ggc tcc act 864
 Gly His Gly Thr His Val Ala Gly Ser Val Leu Gly Asn Gly Ser Thr
 275 280 285
 45 aat aaa gga atg gcg cct cag gcg aat cta gtc ttc caa tct atc atg 912
 Asn Lys Gly Met Ala Pro Gln Ala Asn Leu Val Phe Gln Ser Ile Met
 290 295 300
 50 gat agc ggt ggg gga ctt gga gga cta cct tcg aat ctg caa acc tta 960
 Asp Ser Gly Gly Gly Leu Gly Gly Leu Pro Ser Asn Leu Gln Thr Leu
 55

305 310 315 320
 5 ttc agc caa gca tac agt gct ggt gcc aga att cat aca aac tcc tgg 1008
 Phe Ser Gln Ala Tyr Ser Ala Gly Ala Arg Ile His Thr Asn Ser Trp
 325 330 335
 10 gga gca gca gtg aat ggg gct tac aca aca gat tcc aga aat gtg gat 1056
 Gly Ala Ala Val Asn Gly Ala Tyr Thr Thr Asp Ser Arg Asn Val Asp
 340 345 350
 15 gac tat gtg cgc aaa aat gat atg acg atc ctt ttc gct gcc ggg aat 1104
 Asp Tyr Val Arg Lys Asn Asp Met Thr Ile Leu Phe Ala Ala Gly Asn
 355 360 365
 20 gaa gga ccg aac ggc gga acc atc agt gca cca ggc aca gct aaa aat 1152
 Glu Gly Pro Asn Gly Gly Thr Ile Ser Ala Pro Gly Thr Ala Lys Asn
 370 375 380
 25 gca ata aca gtc gga gct acg gaa aac ctc cgc cca agc ttt ggg tct 1200
 Ala Ile Thr Val Gly Ala Thr Glu Asn Leu Arg Pro Ser Phe Gly Ser
 385 390 395 400
 30 tat gcg gac aat atc aac cat gtg gca cag ttc tct tca cgt gga ccg 1248
 Tyr Ala Asp Asn Ile Asn His Val Ala Gln Phe Ser Ser Arg Gly Pro
 405 410 415
 35 aca aag gat gga cgg atc aaa ccg gat gtc atg gca ccg gga acg ttc 1296
 Thr Lys Asp Gly Arg Ile Lys Pro Asp Val Met Ala Pro Gly Thr Phe
 420 425 430
 40 ata cta tca gca aga tct tct ctt gca ccg gat tcc tcc ttc tgg gcg 1344
 Ile Leu Ser Ala Arg Ser Ser Leu Ala Pro Asp Ser Ser Phe Trp Ala
 435 440 445
 45 aac cat gac agt aaa tat gca tac atg ggt gga acg tcc atg gct aca 1392
 50
 55

Asn His Asp Ser Lys Tyr Ala Tyr Met Gly Gly Thr Ser Met Ala Thr
 5 450 455 460
 ccg atc gtt gct gga aac gtg gca cag ctt cgt gag cat ttt gtg aaa 1440
 Pro Ile Val Ala Gly Asn Val Ala Gln Leu Arg Glu His Phe Val Lys
 10 465 470 475 480
 aac aga ggc atc aca cca aag cct tct cta tta aaa gcg gca ctg att 1488
 15 Asn Arg Gly Ile Thr Pro Lys Pro Ser Leu Leu Lys Ala Ala Leu Ile
 485 490 495
 gcc ggt gca gct gac atc ggc ctt ggc tac ccg aac ggt aac caa gga 1536
 20 Ala Gly Ala Ala Asp Ile Gly Leu Gly Tyr Pro Asn Gly Asn Gln Gly
 500 505 510
 tgg gga cga gtg aca ttg gat aaa tcc ctg aac gtt gcc tat gtg aac 1584
 25 Trp Gly Arg Val Thr Leu Asp Lys Ser Leu Asn Val Ala Tyr Val Asn
 515 520 525
 gag tcc agt tct cta tcc acc agc caa aaa gcg acg tac tcg ttt act 1632
 30 Glu Ser Ser Ser Leu Ser Thr Ser Gln Lys Ala Thr Tyr Ser Phe Thr
 35 530 535 540
 gct act gcc ggc aag cct ttg aaa atc tcc ctg gta tgg tct gat gcc 1680
 40 Ala Thr Ala Gly Lys Pro Leu Lys Ile Ser Leu Val Trp Ser Asp Ala
 545 550 555 560
 cct gcg agc aca act gct tcc gta acg ctt gtc aat gat ctg gac ctt 1728
 45 Pro Ala Ser Thr Thr Ala Ser Val Thr Leu Val Asn Asp Leu Asp Leu
 565 570 575
 gtc att acc gct cca aat ggc aca cag tat gta gga aat gac ttt act 1776
 50 Val Ile Thr Ala Pro Asn Gly Thr Gln Tyr Val Gly Asn Asp Phe Thr
 580 585 590
 55

tcg cca tac aat gat aac tgg gat ggc cgc aat aac gta gaa aat gta 1824

Ser Pro Tyr Asn Asp Asn Trp Asp Gly Arg Asn Asn Val Glu Asn Val

600

605

ttt att aat gca cca caa agc ggg acg tat aca att gaa gta cag gct 1872

Phe Ile Asn Ala Pro Gln Ser Gly Thr Tyr Thr Ile Glu Val Gln Ala

615

620

tat aac gta ccg gtt gga cca cag aac ttc tcg ttg gca att gtg aat 1920

Tyr Asn Val Pro Val Gly Pro Gln Asn Phe Ser Leu Ala Ile Val Asn

630

635

640

taa

1923

30 Claims

1. An alkaline protease which has the following physicochemical properties:

- (i) Acting pH range**

acting over a wide pH range of 4-13 and exhibiting, at a pH of 6-12, 80% or more the activity at the optimum pH:

- (ii) Stable pH range**

being stable over a pH range of 6-11 when treated at 40°C for 30 minutes;

- (iii) Isoelectric point**

having an isoelectric point of approximately 8.9-9.1; and

- (iv) Effect of a fatty acid**

casein-degrading activity not being inhibited by oleic acid.

2. An alkaline protease according to claim 1, which has an estimated molecular weight of approximately 43,000 as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

3. An alkaline protease according to claim 1 or 2, which has an amino acid sequence shown by Sequence No. 1 or 2, or such a sequence in which one or more amino acids are deleted, substituted, or added.

- 4. A gene encoding an alkaline protease according to any one of claims 1 through 3.**

5. A microorganism producing an alkaline protease according to any one of claims 1 through 3.
6. A detergent composition containing an alkaline protease according to any one of claims 1 through 3.

5

10

15

20

25

30

35

40

45

50

55

Fig. 1

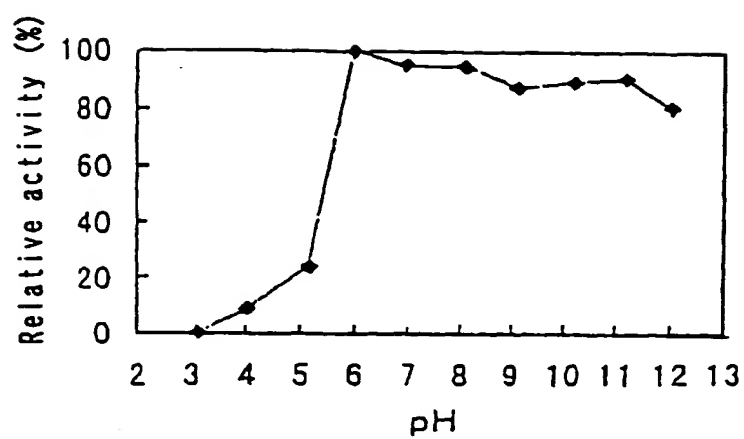


Fig. 2

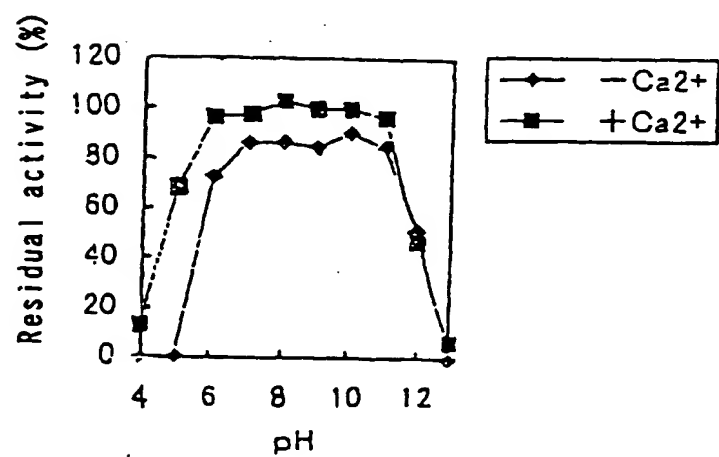


Fig. 3

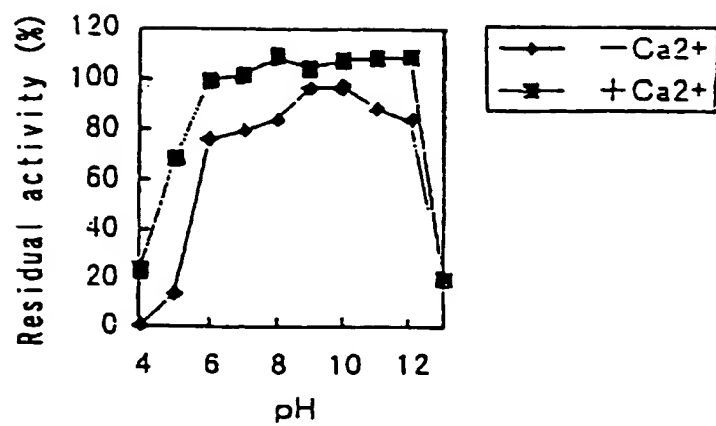


Fig. 4

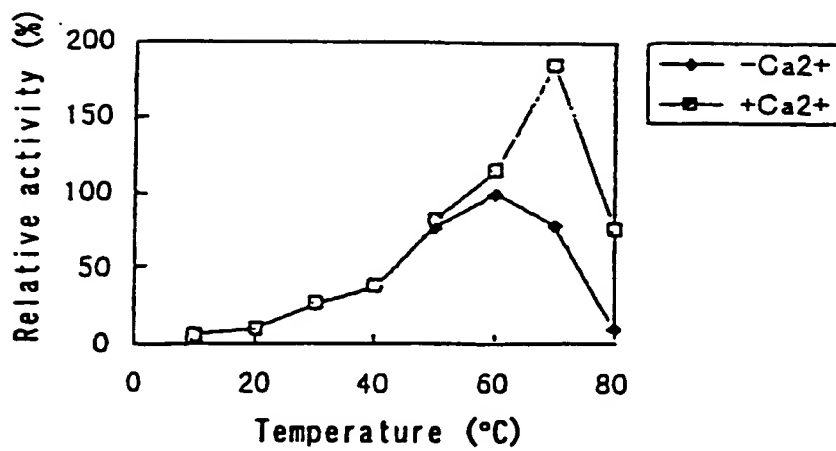


Fig. 5

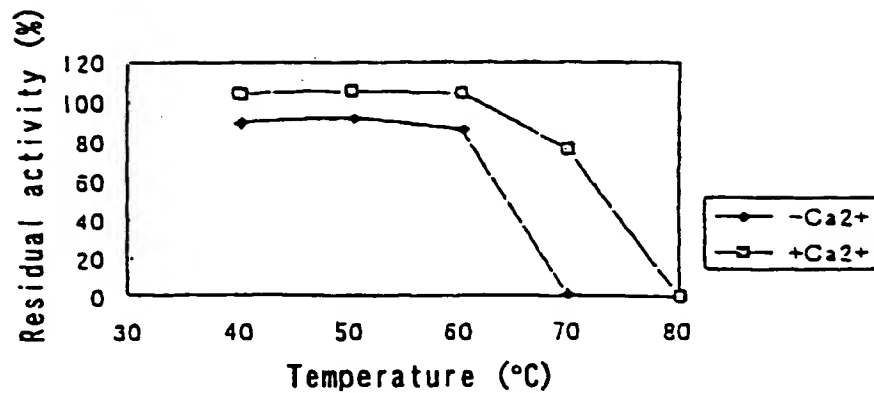


Fig. 6

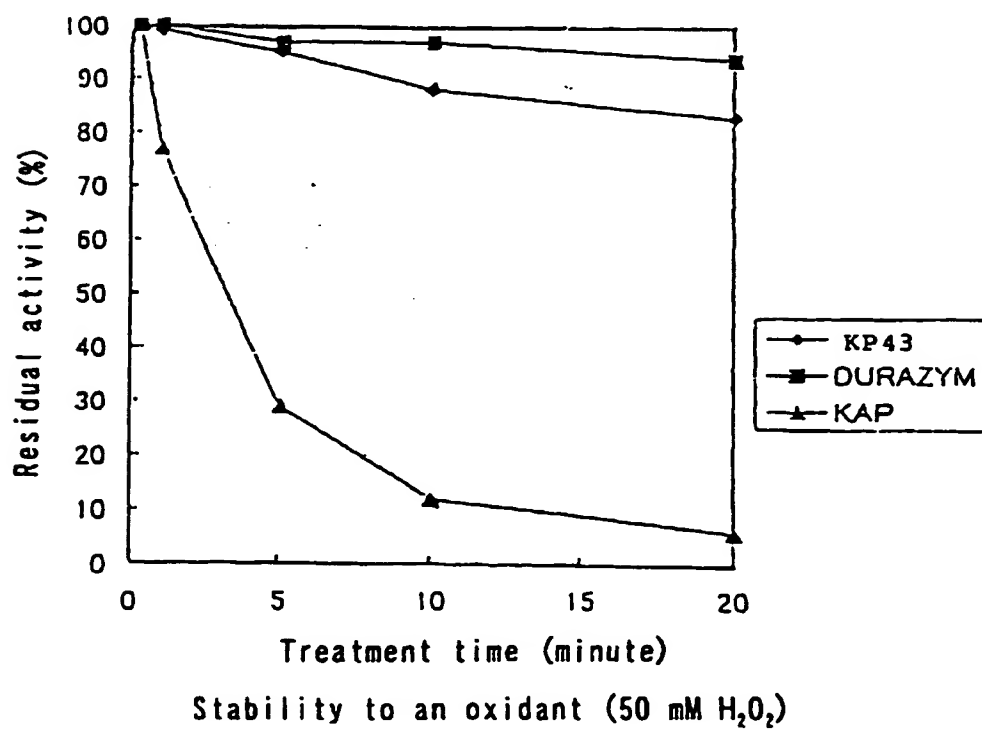


Fig. 7

N-terminal sequence of KP-9860 protease	NOVARHIVKADVAQSSYGLY
N-terminal sequence of 15kDa partially degraded product	GIVKADVAQSSYGL
N-terminal sequence of 18kDa partially degraded product	IKPDVMAPGTYIL
N-terminal sequence of 25kDa partially degraded product	NAITVGATENLRPSEGSYAD
N-terminal sequence of 28kDa partially degraded product	KNDMVILFAAGNEGPN

Fig. 8

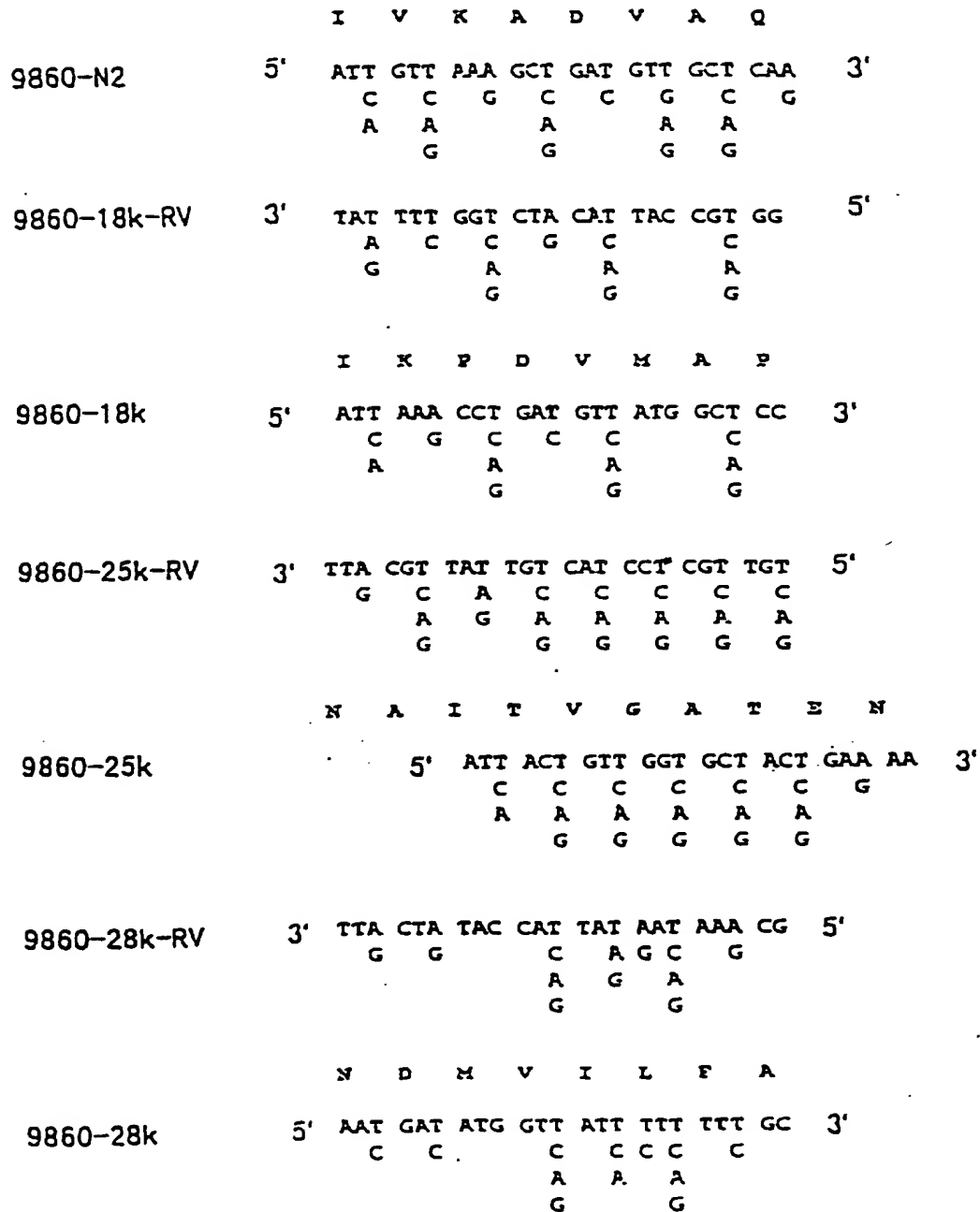
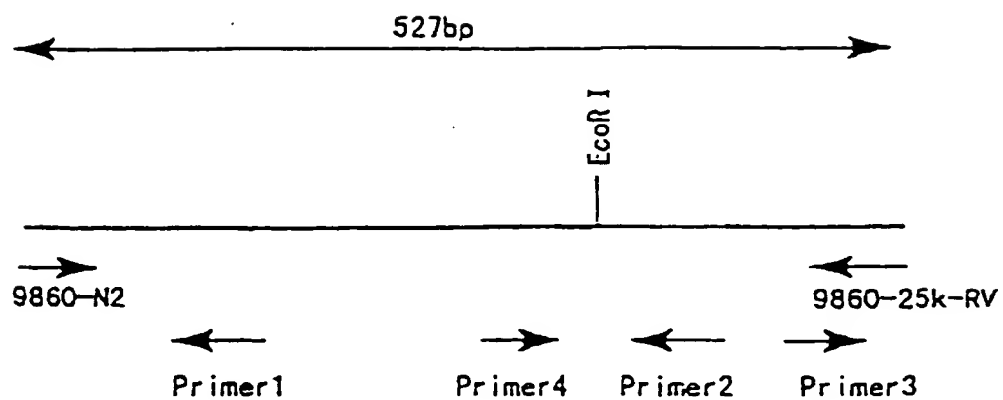


Fig. 9



Primer1 : TCGGCAACTGCGACAATCTGG

Primer2 : TCTGGAATCTGTCGTGTAGGC

Primer3 : AACGGCGGTACCATCAGTGC

Primer4 : GGAGGCTTGCCTTCCAATCTG

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP98/04528

A. CLASSIFICATION OF SUBJECT MATTER Int.Cl ¹ C12N15/57, C12N9/54, C12N1/21, C11D3/386 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int.Cl ¹ C12N15/57, C12N9/54, C12N1/21, C11D3/386 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) BIOSIS (DIALOG), WPI (DIALOG), GenSeq		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of documents, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, 4-197182, A (Lion Corp.), 16 July, 1992 (16. 07. 92) (Family: none)	1-6
A	JP, 6-70765, A (Showa Denko K.K.), 15 March, 1994 (15. 03. 94) (Family: none)	1-6
A	JP, 9-121855, A (TOTO Ltd.), 13 May, 1997 (13. 05. 97) (Family: none)	1-6
A	JP, 5-211868, A (Hokkaido Sugar Co., Ltd.), 24 August, 1993 (24. 08. 93) (Family: none)	1-6
A	JP, 9-121856, A (Kao Corp.), 13 May, 1997 (13. 05. 97) (Family: none)	1-6
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "A" document member of the same patent family	
Date of the actual completion of the international search 16 December, 1998 (16. 12. 98)		Date of mailing of the international search report 22 December, 1998 (22. 12. 98)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.		Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)